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13. Abstract ***(Maximum 200 Words)*** Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivators modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) as a coactivator of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. We propose to explore this by developing animal models for overexpression and loss of function of E6-AP and then relate these observations to the clinical setting by studying the expression patterns of E6-AP in various human breast tumors. In this report, we report that we have successfully generated E6-AP overexpression models and an E6-AP null mouse line. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compared to that of control mammary gland. These mice exhibit increased ductal branching and alveolar buds. However, the overexpression of transgene E6-AP and loss of function of E6-AP have no significant effects on the pregnant mammary glands. Additionally, we also show that E6-AP modulates the p53 expression in these animals. E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Our data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ligase activity of E6-AP. To study the expression profile of E6-AP in human breast tumors, we examined 100 different human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. Furthermore, our data also demonstrate that ~80% human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (*1-6*). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (*7-10*). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, estrogen (ER) and progesterone (PR) receptors (*11-13*). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (*11-13*).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (*14-19*). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (*20-22*). A number of coactivators have been cloned to date, including SRC-1 (*23*), TIF2 (GRIP1) (*24-27*), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (*28-32*), PGCs (*33*), SRA (*34*), CBP (*35-37*) and **E6-associated protein (E6-AP)** (*38*) etc. and this list is growing rapidly. To date more than 70 different coactivator proteins have been identified.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (*39, 40*). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone acetyl transferase, HAT, activity (*14, 16, 28, 30, 41-43*) and members of SWI/SNF complex contain an ATPase activity (*44-47*). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic

interactions between histones and their substrate DNA (*42-48*). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (*17, 49-51*). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (*42-47*). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes (UBCs) and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (*16, 38, 52-54*). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (*55-57*). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (*38*). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (*58, 59*). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (*60-62*). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and E2 ubiquitin conjugating enzymes, UBCs. The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (*63, 64*). The carboxyl-terminal 350 amino acids (aa) of E6-AP contains a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date. The extreme carboxyl-terminal 100 aa contains the catalytic region of E6-AP, which transfers ubiquitin to the protein targeted for degradation (*60,*

61). We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP. It has been shown that the conserved cysteine (C) 833 residue in E6-AP forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the proteins targeted for ubiquitination. The mutation of C833 to alanine (A) or serine (S) has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (*60, 61*). In cotransfection studies, we showed that an E6-AP bearing a C-to-S mutation at the critical site was still able to coactivate steroid hormone receptors. Furthermore, our data also indicate that the catalytic function located within the *hect* domain of E6-AP is not necessary for the ability of E6-AP to interact with and coactivate steroid hormone receptor function, further confirming that the ubiquitin-ligase activity of E6-AP is not necessary for E6-AP to function as a coactivator (*38*). These findings indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity (*38*).

It has been shown that altered expression of one nuclear receptor coactivator; AIB1 contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (*28*). Recenly, it has also been shown that another steroid receptor coactivator, SRA is also elevated in breast tumors (*65*). Furthermore, recently, we have also shown that E6-AP is overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (*66*).

The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this by developing animal models for overexpression and loss of function of E6-AP. To relate the observations obtained from these animal models to the clinical setting, we also proposed to study the expression patterns of E6-AP in various human breast tumor biopsy samples. In this progress report, we report that we have successfully generated two E6-AP overexpression models. In these models, we have overexpressed wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP (C833S) in the mammary epithelium of mouse by using the mouse mammary tumor virus (MMTV) promoter. In order to study the effect of loss of function of E6-AP on the normal mammary gland development and mammary gland tumor development, we have acquired an E6-AP null mouse line. These models will be helpful in understanding the role of E6-AP in the development and progression of breast tumors. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compared to that of the control mammary gland. These mutant mice exhibit increased ductal branching and alveolar buds. As mentioned above E6-AP contains both the coactivation activity and ubiquitin-protein ligase activity. In order to identify the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of breast tumors, we also generated a mouse transgenic line, which overexpressed ubiquitin-protein ligase defective and coactivation function intact E6-AP (C833S) in the mammary epithelium. Like E6-AP null mice, the overexpression of ubiquitin-protein ligase defective mutant E6-AP in mammary gland results in an overly developed mammary gland compared to that of the control mammary gland. Furthermore, these mice also

exhibit increased ductal branching and alveolar buds. These data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ubiquitin-protein ligase activity of E6-AP. Additionally, we also show that the levels of p53 protein are modulated by E6-AP in these animal models. The p53 expression is low in animals that overexpress wild-type E6-AP in the mammary gland compared to that of control animals. In contrast, the p53 expression is high in E6-AP knockout animals and the animals that overexpress ubiquitin-protein ligase defective E6-AP protein compare to that of control animals. These data suggest that in mammary gland, the E6AP protein degrades the p53 protein. This result is consistent with the previously published in vitro experiments that suggest that E6-AP promote the degradation of p53 via the ubiquitin-proteasome pathway *(60)*. In order to study the expression profile of E6-AP in human breast tumors, we also examined 100 different human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. Furthermore, our data also demonstrate that ~80% of human breast tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

Body

In this original proposal, we hypothesized that E6-AP is an important modulator of the steroid hormone receptor mediated signal transduction pathway, and in cell growth and cycle control that are functionally significant in the development of breast cancer. In order to test this hypothesis we propose following objectives:

- **Development and analysis of animal models for the overexpression of wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP in the mammary gland.**
- **Analysis of an animal model for loss of E6-AP expression in the mammary gland.**
- **Expression analysis of endogenous E6-AP, ER and p53 in human breast tumor biopsy samples.**

Development and analysis of an animal model for the overexpression of E6-AP in the mammary gland.

In order to test the effect of overexpression of E6-AP on the development of normal mammary gland and development of mammary tumors, we have successfully generated new transgenic mouse models. These models overexpress wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP proteins in the mammary epithelium. In order to target the expression of E6-AP protein to the mammary epithelium we have utilized the MMTV promoter. Several other investigators have also successfully used this promoter to target expression of transgenes to the mammary gland (*67-70*).

Task 1. Design and generation of transgenic vectors

To overexpress wild-type E6-AP and ubiquitin-protein ligase defective mutant E6-AP in mouse mammary gland, transgenic expression vectors were generated (Fig. 1A). These vectors contain

the MMTV promoter fused to either wild-type human E6-AP cDNA or to the ubiquitin-protein ligase defective (C833S) mutant E6-AP cDNA. To enhance the expression of the transgenes, the rabbit beta-globin gene fragment containing exon II, intron II, exon III and poly A signal sequences were also incorporated into the transgene vectors. These vectors contain the requisite splice acceptor and donor sites for maximum transgene expression. In order to distinguish transgene expression from endogenous mouse E6-AP, we fused flag tag to the amino-terminus of the E6-AP. In the original proposal, we proposed to use the Anti-Express tag but it turns out that Anti-Express tag is not sensitive enough to detect the expression of E6-AP using Western blot analysis. Therefore, we were forced to incorporated flag tag in the E6-AP transgenic expression vector instead of Anti-Express tag. Using flag tag we have successfully detected the expression of wild-type E6-AP (Fig. 1B) and ubiquitin-protein ligase defective mutant E6-AP by Western blot analysis (data not shown).

The MMTV-flag-E6-AP transgenic expression vector was constructed as follows: initially a linker (5'-AATTCCCCGGG-3' and 5'-AATTCCCGGGG-3') containing the internal XmaI site was inserted into the E.CoRI site of the MMTVkBpA expression vector and resultant plasmid was named MMTVkBpAXmaI. To insert flag-tagged E6-AP into the MMTVkBpA expression plasmid, the full length E6-AP cDNA was amplified by polymerase chain reaction (PCR) with the primers containing flag tag sequences 5'-TCCCCCCGGGATGGACTACAAGGACGACGATGACAAGGAAGCCTGCACGAATGAG-3' (upper strand) and 5'-TCCCCCCGGGTTACAGCATGCCAAATCCTTTGGCATACGTGATGGCCTT-3' (lower strand). The PCR product was digested with XmaI and cloned into the corresponding site of the MMTVkBpAXmaI. After sub cloning the PCR amplified cDNA of E6-AP was sequenced and was found to be correct in sequence and reading frame. In order to make MMTV-mutant E6-AP (C833S), the full length cDNA of ubiquitin-protein ligase defective mutant E6-AP in which C833 was changed to S was amplified by PCR with the above mentioned primers and the PCR product was digested with XmaI and cloned into the corresponding site of the MMTVkBpAXmaI. After sub cloning the PCR amplified cDNA of E6-AP was sequenced and was found to be correct in sequence and reading frame.

In order to determine whether the MMTV-flag-E6-AP expression vectors was able to express full length E6-AP protein, this vector was transiently transfected into HeLa cells and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibodies. As shown in Fig. 1B, MMTV-flag-E6-AP expression vector was able to express full length E6-AP protein compared to that of control vector, which does not contain flag-E6-AP cDNA. Furthermore, Fig. 1B also suggest that anti-flag antibody is able to detect E6-AP expression by Western blot. Similarly, MMTV-mutant E6-AP expression vector was also able to express full-length ubiquitin-protein ligase defective mutant E6-AP protein (data not shown).

Next we asked whether the wild-type flag-E6-AP protein and the ubiquitin-protein ligase defective mutant E6-AP protein functions as coactivator of nuclear hormone receptors. Previously, we have shown that E6-AP acts as a coactivator of PR and ER in cells. To test the coactivation function of wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP proteins, the E6-AP expression plasmids along with receptor expression and reporter plasmids were cotransfected into HeLa cells. Then cells were treated with appropriate hormones

and the activity of the reporter gene was measured. Fig. 2 suggests that in the absence of ligand, PR has a minimal effect on reporter gene expression either in the absence or in the presence of E6-AP. Addition of hormone increases the reporter gene activity in the absence of E6-AP; when flag-E6-AP was coexpressed with PR, the activity of PR was further stimulated by 4 to 5-fold. Similarly, the flag-E6-AP protein was also able to enhance the ER activity in HeLa cells (Fig. 3). Like wild-type E6-AP, the ubiquitin-protein ligase defective mutant E6-AP was also able to enhance both the PR and ER activities. These data suggest that wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP are functional and both act as coactivators of PR and ER.

Task 2. Generation of transgenic animals

After establishing that the wild-type flag-tagged E6-AP protein is intact and biologically functional, the transgene was released from the transgenic expression vector by digesting MMTV-flag-E6-AP vector with NotI and KpnI enzymes. After purification from the vector backbone, the transgene DNA was extracted with phenol-chloroform and ethanol pracipitated. After precipitation, the transgene DNA was suspended in injection buffer and microinjected into fertilized FVB one-cell embryos. The injected embryos were then implanted into the oviducts of pseudopregnant recipient mothers. Once, animals were born, the transgenic founders were identified by PCR and/or Southern blot analysis.

Task 3. Identification of transgenic founders

In order to identify the transgenic lines, we have developed a PCR screening method. PCR screening is faster and cheaper compared to Southern blot screening. To develop PCR screen we designed 2 pairs of primer sets. The locations of these primers in the transgene are shown in Fig. 4. The primers 1 and 2 will generate a 385bp fragment and while the primers 3 and 4 will generate 450bp fragment (Fig. 4). The transgene-negative animals will not generate these bands. The sequence of the primers are as follows: primer 1, 5'-TGCTAACCATGTTCATGCC-3'; primer 2, 5'-CTCAGAGCAGGAGTTGTTGGG-3'; primer 3, 5'-ATGGACTACAAGGACGACGATG-3' and primer 4 5'-CCGGAAGCTCTGTACC-3'. In order to confirm the PCR result we have also performed Southern blot analysis of transgenic lines (Fig. 5). The lines, which are positive for transgene by PCR method, are also positive by Southern blot analysis (Fig. 5). By using PCR and Southern blot screening methods, we identified two transgene positive founder lines. However, only one founder was able to transmit the transgene to the offsprings. After another round of injection, we have identified two more transgenic lines. In total we have three transgene lines which can transmit transgene to their offspring. Finally, founders were bred with wild-type FVB mice to generate female mice for further analyses.

Task 4 and 5. Breed founders and analysis of expression patterns of transgene

Next, we analyzed transgenic lines for the expression of transgene human E6-AP in the mouse mammary gland. In order to confirm whether the human E6-AP transgene is expressed in mouse mammary gland, we analyzed the mammary glands of 8 weeks old virgin female mice by immunohistochemistry using an anti-E6-AP specific antibody obtained from Dr. N. J. Maitland.

As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. In order to study the expression profile of transgene, the mammary glands from 8 weeks old wild-type non-transgenic and transgenic virgin female mice were microdissected, fixed in 10% formalin and processed for immunohistochemistry studies using an anti-E6-AP specific polyclonal antibody produced in rabbit. This antibody recognized both human E6-AP and endogenous mouse E6-AP. As shown in Fig. 6, the human E6-AP transgene is highly expressed in the transgenic line E106 (Fig. 6A) and moderately expressed in transgenic lines E95 (Fig. 6B) and E37 (data not shown). Furthermore, transgene is specifically targeted to the mammary epithelium. Fig. 6 also suggests that the expression of endogenous mouse E6-AP is very low in the mammary gland. The control sections incubated with normal serum showed no signal (data not shown). We have also analyzed the expression of transgene by using anti-flag antibody. This analysis also demonstrates that human E6-AP transgene is highly expressed in the transgenic line E106 and moderately expressed in transgenic lines E95 and E37 (data not shown). These results were also further confirmed by Western blot analysis (data not shown).

To determine the tissue specificity of transgene expression, total cellular extracts were prepared from various transgenic tissues (brain, liver, mammary gland and heart) and the expression pattern of transgene was compared with that of non-transgenic tissues by Western blot analysis using anti-E6-AP antibody. As expected endogenous E6-AP is highly expressed in brain and liver (Fig. 7). The expression of E6-AP is moderate in heart. However, low expression of endogenous E6-AP was detected in mammary gland. Fig. 7 shows that transgene human E6-AP is selectively overexpressed in mammary gland. However, the transgenic line E37 also showed some expression of transgene in lung (data not shown). The expression profile of our transgene is in agreement with that of published expression profile of other MMTV-transgenes. Again, transgenic line E106 showed highest expression of transgene and transgenic line E95 showed moderate expression of transgene (Fig. 7).

Task 6. Morphological and histological analysis of transgenic mammary glands

In order to analyze for physiological perturbations that could be attributed to the overexpression of E6-AP, we performed whole-mount analysis of transgenic and age-matched wild-type non-transgenic mammary glands at different stages of development, (a) virgin (8 weeks old, 12 weeks old and 68 weeks old); (b) pregnant; (c) lactation; and (d) involution (15 days involuting and 8 weeks involuting) stages. In newborn mice, the mammary gland is comprises of a few ducts and it undergoes extensive growth post-natally. During puberty, the elongation and arborization of the ducts progress gradually into the surrounding mammary fat pad under the influence of gonadal hormones and terminate at the limits of the fat pad. With each subsequent estrous cycle, the lateral ductal branches subdivide progressively and give rise to small alveolar buds. During pregnancy additional ductal branching occurs and extensive lobular-alveolar proliferation gradually results in the complete filling of the fat pad at parturition (*71*). The whole mount analyses of 8 weeks old, 12 weeks old and 68 weeks old virgin mammary glands from human E6-AP transgenic lines (E95 and E106) and wild-type non-transgenic lines were performed. As shown in Fig. 8, 9 and 10, overexpression of transgene human E6-AP results in impaired mammary gland development compared to wild-type non-transgenic litttermates. The transgenic mammary glands failed to invade the entire fat pad. In contrast, the wild-type gland was able to

invade the entire fat pad. In this study we used lymph node as a reference point to evaluate ductal outgrowth. In each group at least six animals were analyzed.

Next, we ask whether overexpression of transgene human E6-AP has any effect on the pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from transgenic and non-transgenic mice. As shown in Fig. 11, the overexpression of human E6-AP has no significant effect on the pregnant mammary gland. The phenotype of the pregnant transgenic and non-transgenic mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of overexpression of transgene human E6-AP on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both transgenic and non-transgenic mice. Fig. 12 suggests that like pregnant glands, the transgene have no significant effect on the involution process during first 15 days. The involution is identical in transgenic and non-transgenic glands during first 15 days. In contrast, whole mount analysis of 8 weeks old involuting transgenic and non-transgenic mammary glands suggest that the transgenic gland involute faster than nontransgenic gland (Fig. 13). Like virgin transgenic mammary gland, the 8 weeks old involuting transgenic gland is smaller compared to that of non-transgenic gland (Fig. 13).

Task 7. Analysis of p53 in transgenic mammary glands

Previously, it has been suggested that E6-AP promotes the degradation of p53 via the ubiquitin-proteasome pathway *(60)*. Therefore, we ask whether the levels of p53 are altered in the mouse transgenic lines that overexpress wild-type E6-AP. In order to study the expression of p53 in mouse mammary gland, we analyzed the mammary glands of 12 weeks old virgin female mice by immunohistochemistry using an anti-p53 specific antibody. As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. Fig. 14 suggests that normal wild-type non-transgenic animals exhibit low expression of p53 in the mammary gland. However, the expression of p53 is reduced in the mammary glands of transgenic animals that overexpress wild-type E6-AP (Fig. 14) suggesting that in mammary gland E6-AP also promotes the degradation of p53 via the ubiquitin-proteasome pathway.

Analysis of an animal model for loss of E6-AP expression in the mammary gland.

The second aim of this proposal is to test the effects of loss of steroid hormone receptor coactivator, E6-AP, on the normal development of mammary gland and mammary gland tumors. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line in our laboratory. Dr. Aurther Beaudet at the Baylor College of Medicine generated this line (*72*).

Task 8. Screening and breeding of E6-AP null mutant mice

To screen for the E6-AP null animals, we have also developed a PCR screening method. In order to develop the PCR screening method, we have designed 3 primers, which can differentiate

between wild-type and null E6-AP locus. The sequence of the primers are: primer 1, 5'-ACTTCTCAAGGTAAGCTGAGCTTGC-3'; primer 2, 5'-GCTCAAGGTTGTATGCCTTGGTGCT-3' and primer 3, 5'-TGCATCGCATTGTCTGAGTAGGTGTC-3'. By using these 3 primers, we have successfully amplified the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type animals have only band of 750 bp, whereas homozygous E6-AP null animals have only 350 bp amplified fragment and heterozygous animals contain both bands (Fig. 15).

Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands

In order to study the consequences of the loss of E6-AP expression on mammary gland development, the whole mount analyses of 8 weeks old and 12 weeks old virgin mammary glands from E6-AP null and wild-type mice were performed. As shown in Fig. 16 and 17A and B, loss of E6-AP results in increased lobular-alveolar buds compare to wild-type normal mammary glands. The E6-AP null mammary glands are more developed and the degree of mammary gland development is similar to that of a 5-10 days pregnant wild-type mammary gland. Increased lobular-alveolar buds in E6-AP null mammary glands compare to that of wild-type mammary glands are clearly visible at higher magnification in Fig. 17B.

Next, we ask whether loss of E6-AP expression has any effect on pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from E6-AP null and normal wild-type mice. As shown in Fig. 18, the loss of E6-AP expression has no significant effect on the pregnant mammary glands. The phenotype of the pregnant E6-AP null and wild-type mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of loss of E6-AP expression on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both E6-AP null and wild-type mice. Fig. 19A and B suggests that like pregnant glands, the loss of E6-AP expression have no significant effect on the involution process. The involution is identical in transgenic and non-transgenic glands.

Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands

As mentioned above it has been shown that E6-AP promotes the degradation of p53 via the ubiquitin-proteasome pathway *(60)*. Furthermore, it has also been demonstrated that the levels of p53 are high in the brain of E6-AP null animals *(72)*. Additionally, our data from E6-AP transgenic animals suggest that the levels of p53 protein are low in transgenic mammary glands compare to that of normal non-transgenic mammary glands (Fig. 14). Therefore, we also analyzed the expression of p53 in E6-AP null mammary glands. Our data suggest that the levels of p53 are high in the E6-AP null mammary glands compared to that of normal mammary glands (Fig. 20).

Development and analysis of an animal model for the overexpression of ubiquitin-protein ligase defective mutant E6-AP (C833S) in the mammary gland.

E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Identification of the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of mammary tumors would be critical to understand the molecular pathway by which E6-AP exert its effects. In order to identify the role of E6-AP coactivation function versus its ubiquitin-protein ligase activity in the development of normal mammary gland and development of breast tumors, we also generated a mouse transgenic line, which overexpress ubiquitin-protein ligase defective and coactivation function intact E6-AP (C833S) in the mammary epithelium.

Task 11. Generation of transgenic animals

In order to generate mouse transgenic lines that overexpress the ubiquitin-protein ligase defective mutant E6-AP, the transgene was released from the transgenic expression vector by digesting MMTV-mutant-E6-AP vector with NotI and KpnI enzymes. After purification from the vector backbone, the transgene DNA was extracted with phenol-chloroform and ethanol pracipitated. After precipitation, the transgene DNA was suspended in injection buffer and microinjected into fertilized FVB one-cell embryos. The injected embryos were then implanted into the oviducts of pseudopregnant recipient mothers. Once, animals were born, the transgenic founders were identified by PCR and/or Southern blot analysis.

Task 12. Breed founders and analysis of expression patterns of ubiquitin-protein ligase defective mutant E6-AP transgene

In order to confirm whether the ubiquitin-protein ligase defective mutant E6-AP transgene is expressed in mouse mammary gland, we analyzed the mammary glands of 8 weeks old virgin female mice by immunohistochemistry using an anti-E6-AP specific antibody. As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. In order to study the expression profile of transgene, the mammary glands from 8 weeks old wild-type non-transgenic and transgenic virgin female mice were microdissected, fixed in 10% formalin and processed for immunohistochemistry studies using an anti-E6-AP specific polyclonal antibody. As shown in Fig. 19, the ubiquitin-protein ligase defective mutant E6-AP transgene is highly expressed in the transgenic line (Fig. 21). Furthermore, our data also demonsttrate that the ubiquitin-protein ligase defective mutant E6-AP transgene is specifically targeted to the mammary epithelium (data not shown). Furthermore, transgene is selectively overexpressed in mammary gland.

Task 13. Morphological and histological analysis of transgenic mammary glands

In order to analyze for physiological perturbations that could be attributed to the overexpression of ubiquitin-protein ligase defective mutant E6-AP, we performed whole-mount analysis of transgenic and age-matched wild-type non-transgenic mammary glands at different stages of development, (a) virgin (12 weeks old); (b) pregnant; (c) lactation; and (d) involution (15 days involuting and 8 weeks involuting) stages. As shown in Fig. 22A and B, like E6-AP null mice, the overexpression of ubiquitin-protein ligase defective mutant E6-AP in mammary gland results

in an overly developed mammary gland compared to that of the control mammary gland. Furthermore, these mice also exhibit increased ductal branching and alveolar buds. Increased lobular-alveolar buds in mutant E6-AP transgenic mammary glands compare to that of wild-type mammary glands are clearly visible at higher magnification in Fig. 22B. These data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ubiquitin-protein ligase activity of E6-AP.

Next, we ask whether overexpression of ubiquitin-protein ligase defective mutant E6-AP transgene has any effect on the pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from transgenic and non-transgenic mice. As shown in Fig. 23, the overexpression of ubiquitin-protein ligase defective mutant E6-AP has no significant effect on the pregnant mammary gland. The phenotype of the pregnant transgenic and non-transgenic mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of overexpression of ubiquitin-protein ligase defective mutant E6-AP transgene on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both transgenic and non-transgenic mice. Fig. 24 suggests that like pregnant glands, the transgene have no significant effect on the involution process during first 15 days. The involution is identical in transgenic and non-transgenic glands during first 15 days. In contrast, whole mount analysis of 8 weeks old involuting transgenic and non-transgenic mammary glands suggest that the transgenic gland has increased alveolar buds compared to that of nontransgenic gland (Fig. 25). Like virgin E6-AP null mammary gland, the 8 weeks old involuting transgenic gland has more lobular-alveolar buds compared to that of non-transgenic gland (Fig. 25).

Expression analysis of endogenous E6-AP, ER and p53 in human breast tumor biopsy samples.

The third aim of the proposal is to test the expression of endogenous E6-AP, ER and p53 in human breast tumor biopsies. To date we have examined expression levels of E6-AP and ER in 100 different breast tumors and expression of p53 in 20 different tumors.

Task 14. Expression analysis of endogenous E6-AP and p53

To study the expression profile of E6-AP in human breast tumors, to date we have examined 100 different human breast cancer biopsy samples by Western blot and immunohistochemistry using E6-AP specific antibody. Fig. 26 shows the expression of E6-AP in 20 different tumor samples. Majority of the tumors expresses E6-AP. We have also confirmed these results by immunofluorescent method using an anti-E6-AP antibody. Since, E6-AP is an E3 ubiquitin-protein ligase enzyme and recently, we have shown that ER is degraded through the ubiquitin proteasome pathway. Therefore, we also analyzed the expression profile of ER in breast tumors (Fig. 26) and then compare it with that of E6-AP expression (Fig. 26). We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant (Fig. 27).

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. Furthermore, in the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Fig. 26, p53 expression was not detectable in most tumors except tumor number 7, 10, and 13. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53.

We have also examined the expression profile of E6-AP in normal human mammary tissues by immunohistochemistry using anti-E6-AP antibody. Fig. 28 suggests that E6-AP is highly expressed in normal human mammary ducts and almost every epithelial cell express E6-AP protein (Fig. 28).

In order to study the expression profile of E6-AP in human breast tumor samples, we examined the expression profile of E6-AP in different human breast tumor samples and adjacent normal tissues by immunohistochemistry. As mentioned above, E6-AP expression is very high in normal mammary ducts (Fig. 29). However, 80% of human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues (Fig. 29).

Next, we analyze the expression level of E6-AP between different stages of breast cancer by immunohistochemistry and then compare the expression profile of E6-AP within different stages by Wicoxonrank-sum test. This analysis suggests that E6-AP expression is decreased in stage IIB breast tumors (Fig. 30).

Statement of work accomplished/in progress

Task 1. Design and generation of transgenic vectors. **Accomplished.**
Task 2. Generation of transgenic animals that overexpress wild-type E6-AP. **Accomplished.**
Task 3. Identification of transgenic founders. **Accomplished.**
Task 4. Breed founders to assess expression and expand positive lines. **Accomplished.**
Task 5. Analysis of expression patterns of transgene in different development stages of mammary glands. **Accomplished.**
Task 6. Morphological and histological analysis of transgenic mammary glands. **Accomplished.**
Task 7. Analysis of p53 expression in transgenic mammary glands. **Accomplished.**
Task 8. Breeding of E6-AP null mutant mice. **Accomplished**
Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands. **Accomplished.**
Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands. **Accomplished.**
Task 11. Generation of transgenic animals that overexpress ubiquitin-protein ligase defective mutant E6-AP. **Accomplished.**
Task 12. Breed founders and analysis of expression patterns of ubiquitin-protein ligase defective mutant E6-AP transgene. **Accomplished.**
Task 13. Morphological and histological analysis of transgenic mammary glands. **Accomplished.**
Task 14. Expression analysis of endogenous E6-AP, ER and p53. **Accomplished.**

Key Research Accomplishments

- E6-AP transgenic expression vectors have been generated
- Expression analysis of E6-AP from the transgenic vector has been completed
- The biological activity of the wild-type flag-E6-AP and ubiquitin-protein ligase defective mutant E6-AP have been analyzed
- Wild-type E6-AP transgenic mouse lines have been generated
- PCR based screening method for identification of transgenic animals has been developed
- E6-AP transgenic founders have been identified by both PCR and Southern blot
- Expression analysis of transgenes have been analyzed in mammary glands
- The tissue specificity of transgenes have been analyzed
- Analysis of E6-AP transgenic mammary glands has been done in virgin, pregnant and involution stages
- Expression analysis of p53 have been analyzed in transgenic mammary glands
- E6-AP knockout animals have been acquired
- PCR based screening method for identification of E6-AP knockout animals has been developed
- Analysis of E6-AP null mammary glands has been done in virgin, pregnant and involution stages
- Expression analysis of p53 have been analyzed in E6-AP knockout mammary glands
- Ubiquitin-protein ligase defective mutant E6-AP transgenic lines have been generated
- Expression analysis of the ubiquitin-protein ligase defective mutant E6-AP transgenes have been analyzed in mammary glands
- The tissue specificity of the ubiquitin-protein ligase defective mutant E6-AP transgenes have been analyzed
- Analysis of the ubiquitin-protein ligase defective mutant E6-AP transgenic mammary glands has been done in virgin, pregnant and involution stages
- Expression analysis of E6-AP in 100 different tumors has been done
- Expression analysis of ER in 100 different tumors has been done
- Expression profile of E6-AP has been compared with that of ER expression
- Expression analysis of p53 in 20 different tumors has been done
- Expression analysis of E6-AP by immunohistochemistry has been done in different breast tumor samples
- Expression analysis of E6-AP in different tumor stages has been done
- Expression profile of E6-AP has been compared within different tumor stages

Reportable Outcomes

1. X. Gao and **Z. Nawaz**. 2002. Role of steroid receptor coactivators and corepressors of progesterone receptors in breast cancer. *Breast Cancer Res.* 4: 40-44. (see appendix 2).

2. X. Gao, B. W. Loggie and Z. Nawaz. 2002. The roles of sex steroid receptor coregulators in cancer. *Molecular Cancer* 1: 1-7. (see appendix 2).

Conclusions

We have successfully generated an E6-AP overexpression model. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compare to that of controls mammary gland. These mice exhibit increased ductal branching and alveolar buds. E6-AP possesses two independent and separable functions: coactivation and ubiquitin-protein ligase activity. Our data suggest that increased ductal branching and alveolar branching in E6-AP null mice are results of loss of ligase activity of E6-AP. Furthermore, we also demonstrated that the levels of p53 are regulated by E6-AP in the mammary glands of transgenic and E6-AP knockout animals. In order to study the expression profile of E6-AP in human breast tumors, we examined 100 different human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. Furthermore, our data also demonstrate that ~80% human tumors exhibited decreased level of E6-AP expression compared to that of normal mammary tissues. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Appendices

1. Figures 1-30

2. 2 Manuscripts

Appendix 1

Figures 1-30

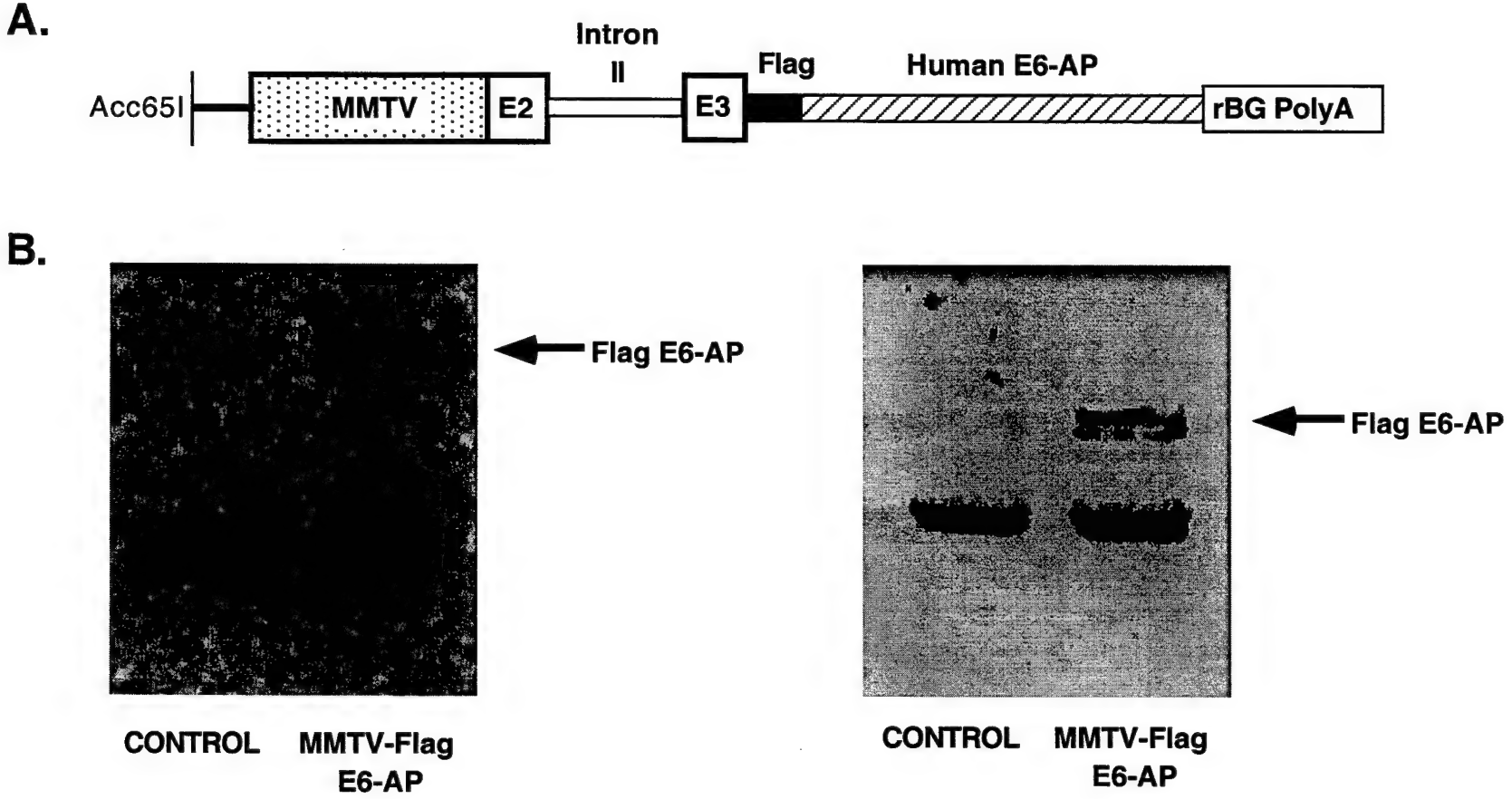


Figure 1: Generation and characterization of MMTV-E6-AP transgene. A. The MMTV-E6-AP construct contains the MMTV promoter and the full-length human E6-AP cDNA, fused to the exon II (E2), intron II, exon III (E3) and the rat beta-globin gene polyadenylation signal (rBG PolyA). **B.** HeLa cells were transiently transfected with either control plasmid or MMTV-Flag-E6-AP expression plasmid and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibody.

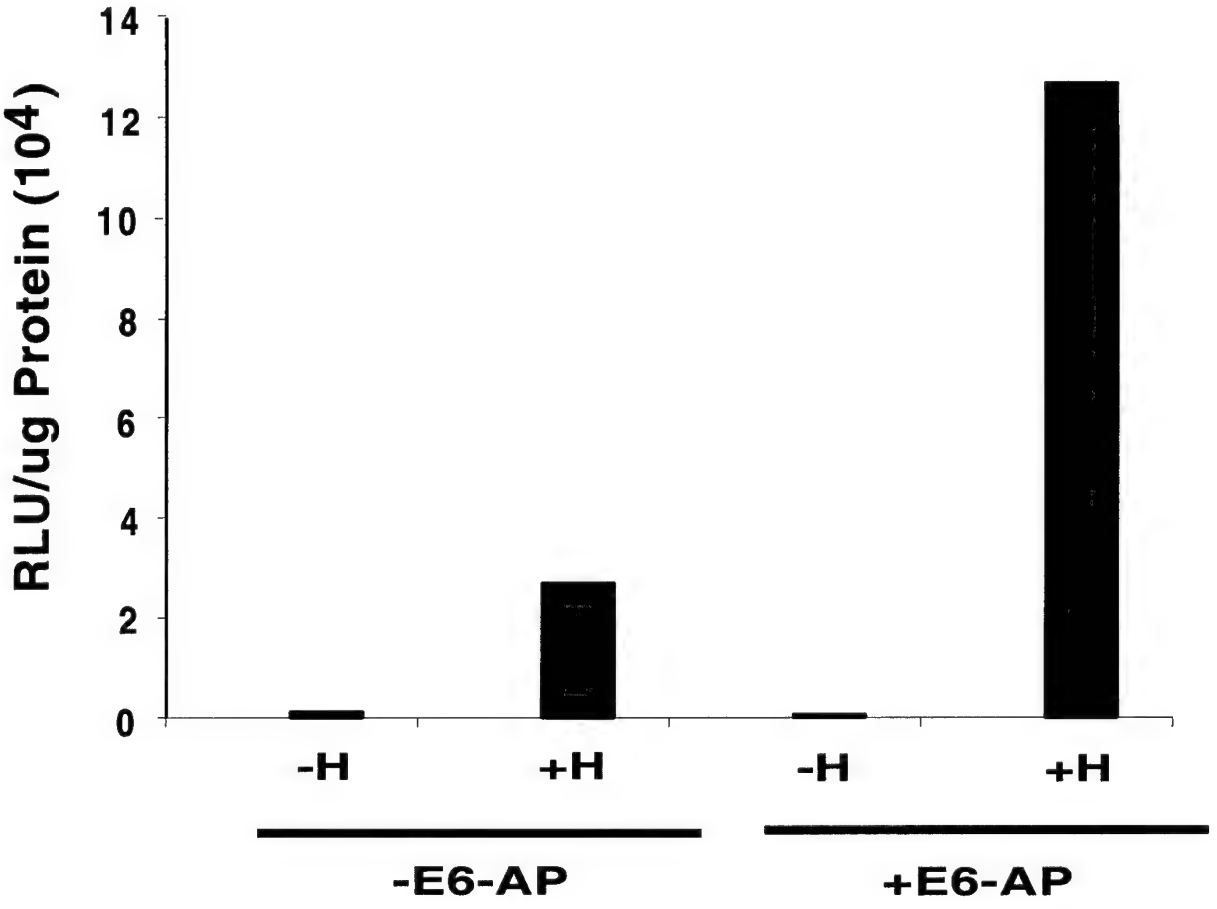


Figure 2: MMTV-Flag-E6-AP Coactivates PR Activity. Hela cells were transiently transfected with progesterone receptor expression plasmid and progesterone-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M progesterone (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

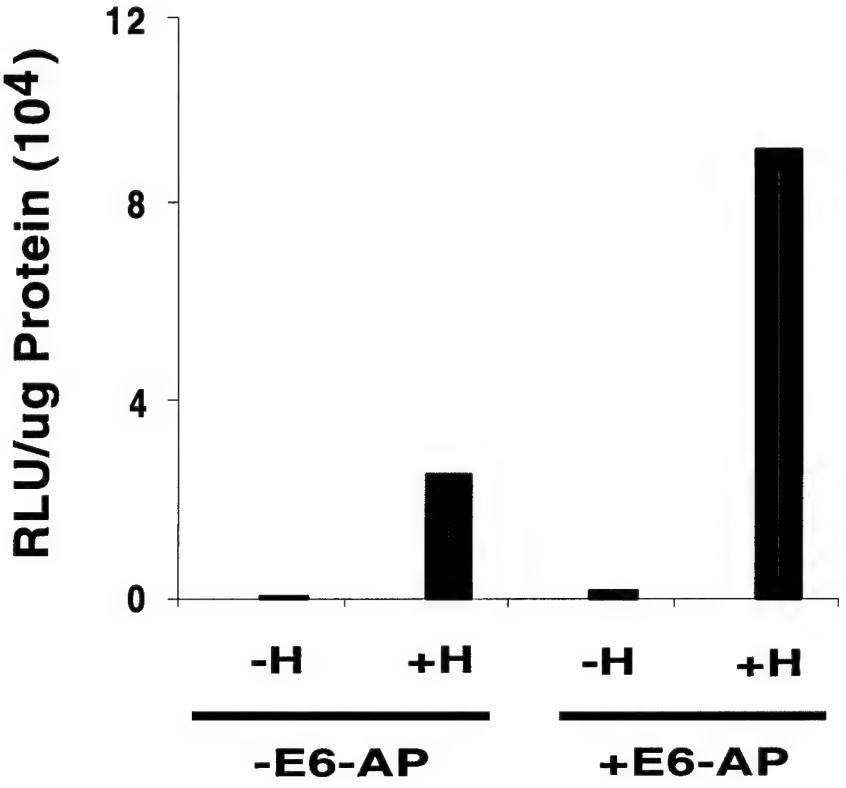


Figure 3: MMTV-Flag-E6-AP Coactivates ER Activity. Hela cells were transiently transfected with estrogen receptor expression plasmid and estrogen-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M estradiol (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

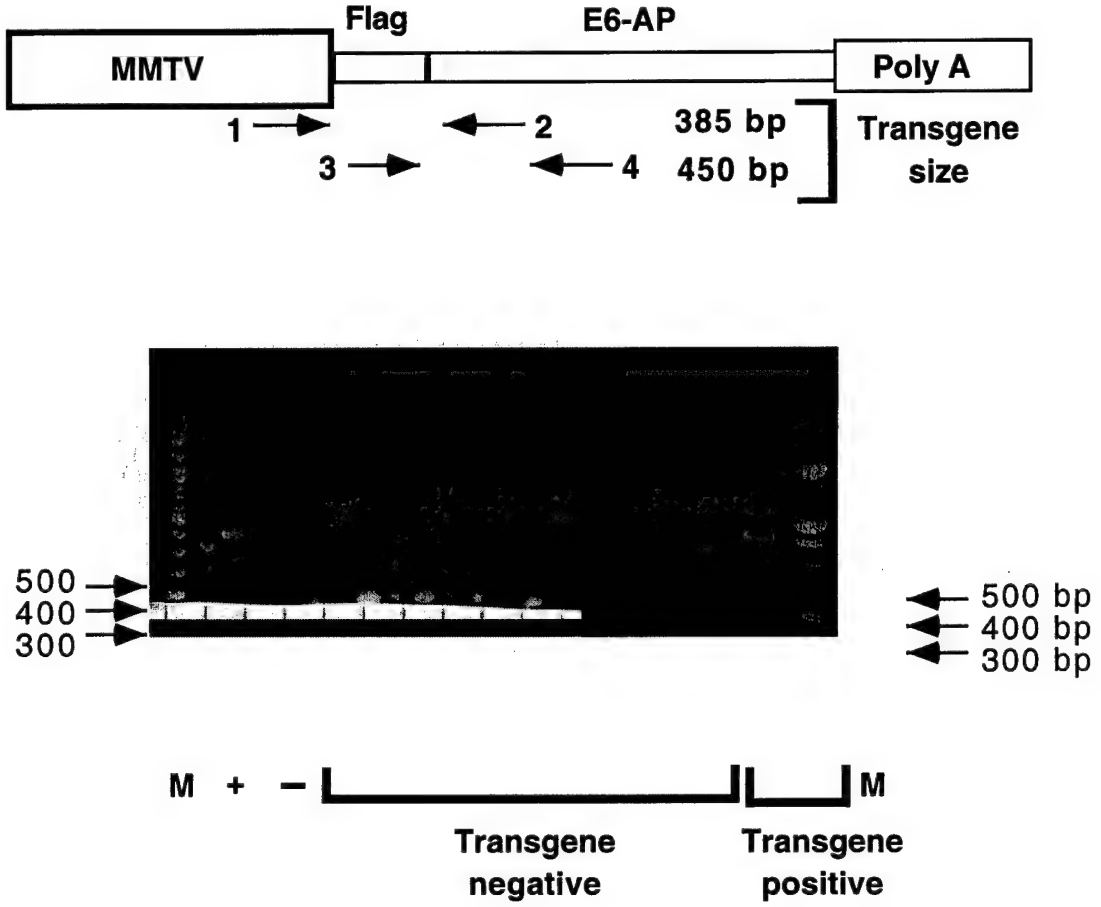


Figure 4: Screening of E6-AP transgenic lines by PCR method.
In order to identify the transgenic lines, a PCR screening method was developed . To develop PCR screen 2 pairs of primer sets were designed. The locations of these primers in transgene are shown in by arrows. The primers 1and 2 amplify a 385bp fragment and the primers 3 and 4 generate 450bp fragment. The transgene negative animals did not generate these bands.

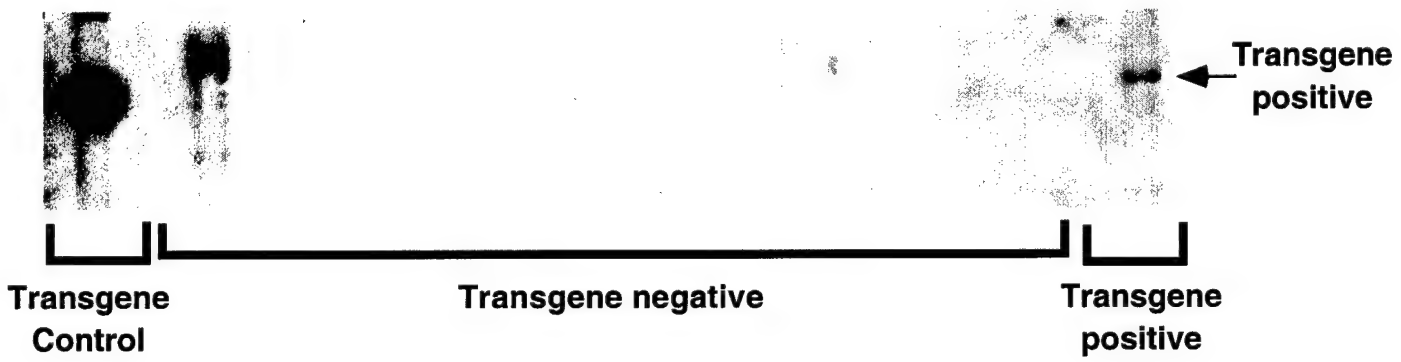
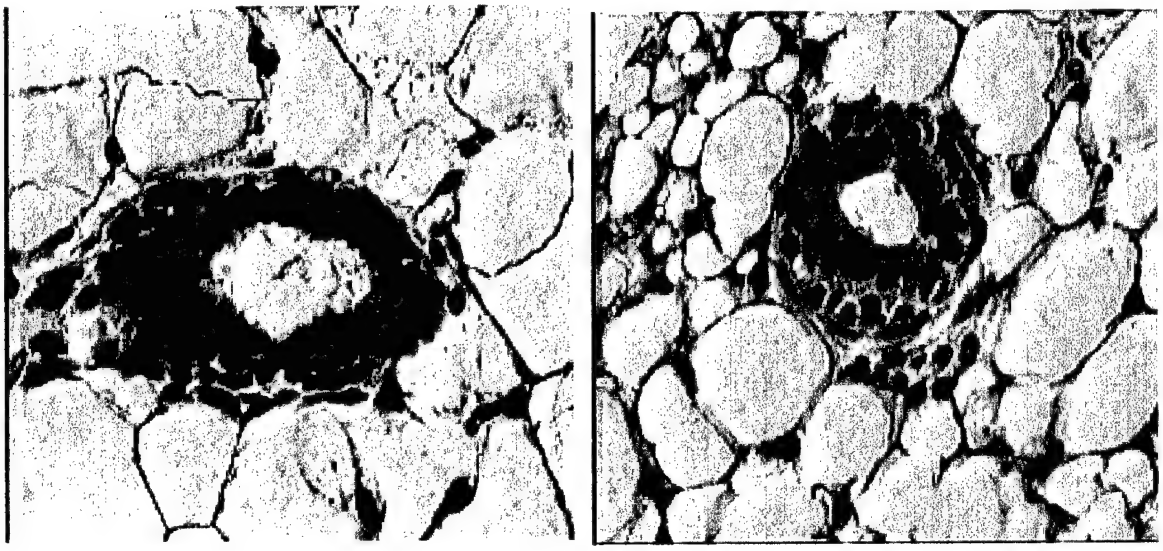


Figure 5: Screening of E6-AP transgenic lines by Southern blot.
Total genomic DNA was isolated from mice tails. The Southern blot was performed by using the 700 bp (BamHI-E.CoRI) long fragment of E6-AP as probe. The genomic DNA was digested with BamHI and BglII.

A.



B.

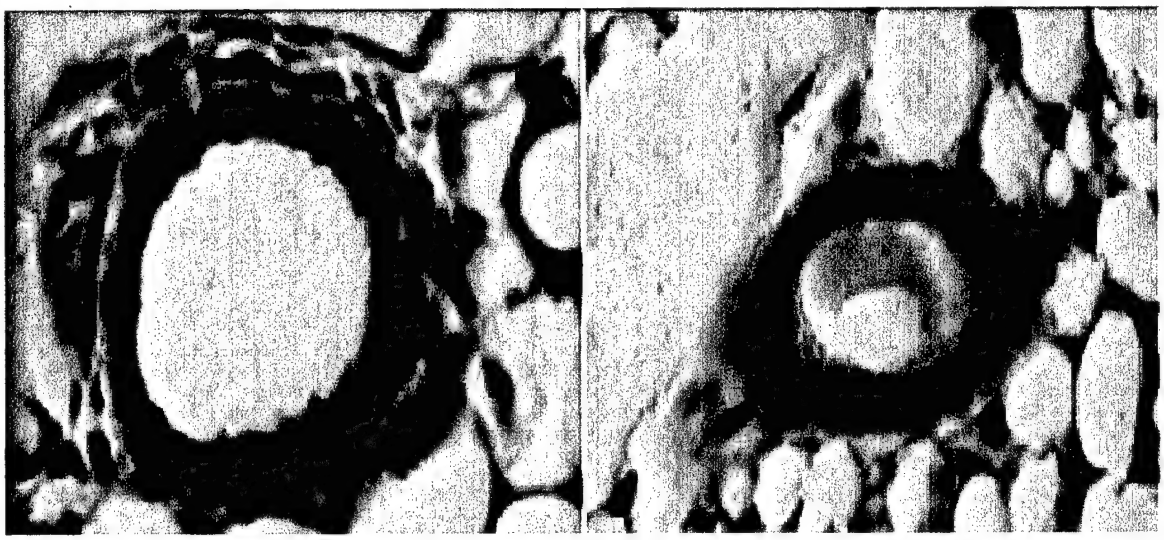


Figure 6: Expression analysis of transgene (MMTV-E6-AP) in the mouse mammary glands. E6-AP expression in the mouse mammary glands was analyzed by immunohistochemistry using an anti-E6-AP antibody. Posit signal is seen as dark (brown) spots. WT, Wild-type mammary gland; TG, E6- transgenic mammarygland. **A.** Transgenic line E106. **B.** Transgenic line E95

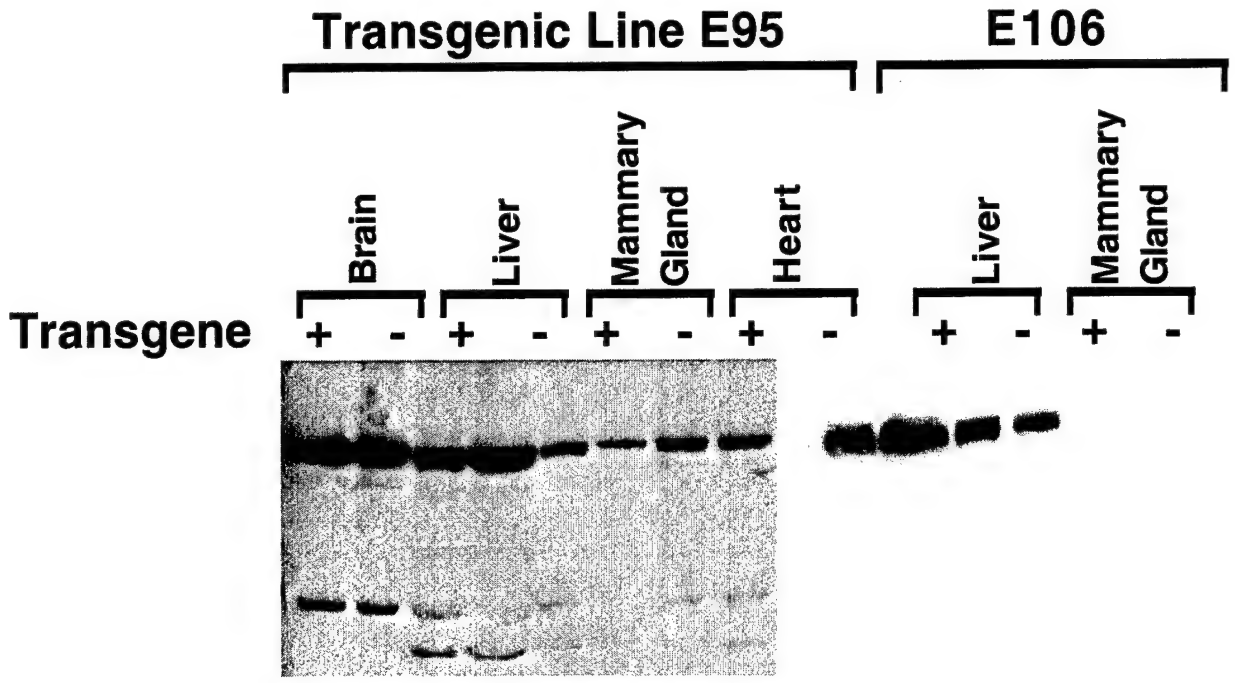


Figure 7: MMTV-driven E6-AP transgene preferentially overexpressed in mammary gland. Expression analysis of endogenous E6-AP and MMTV-driven human E6-AP transgene was performed in various mouse tissues such as brain, liver, mammary gland, heart etc. by Western blot using E6-AP specific antibody. MMTV-driven E6-AP transgene is specifically overexpressed in mammary gland.

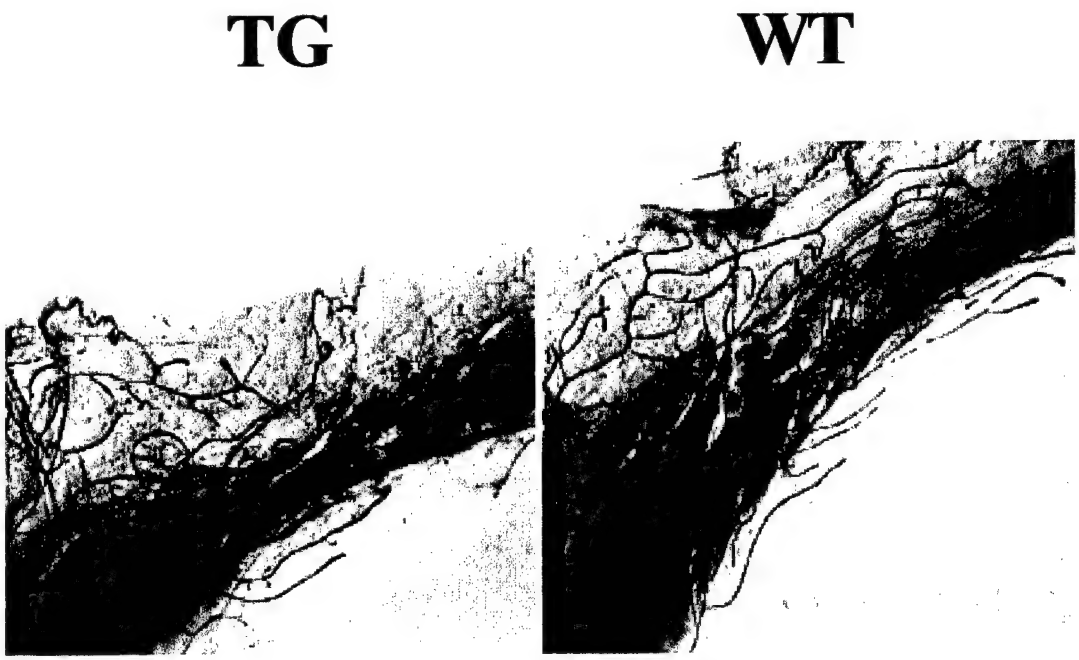


Figure 8: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 8 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

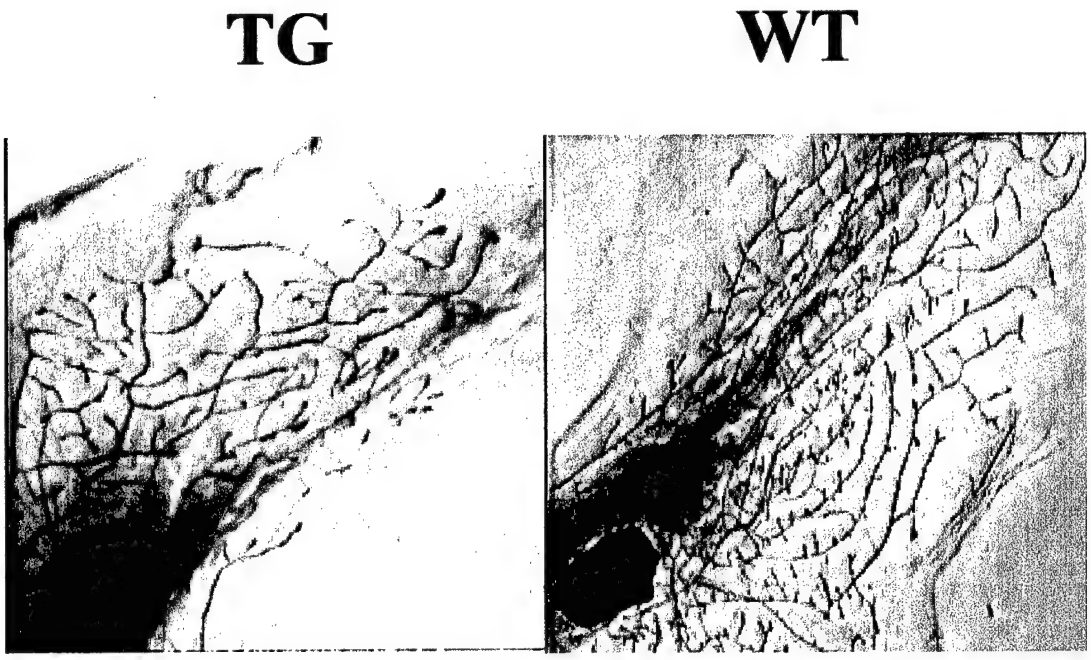


Figure 9: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

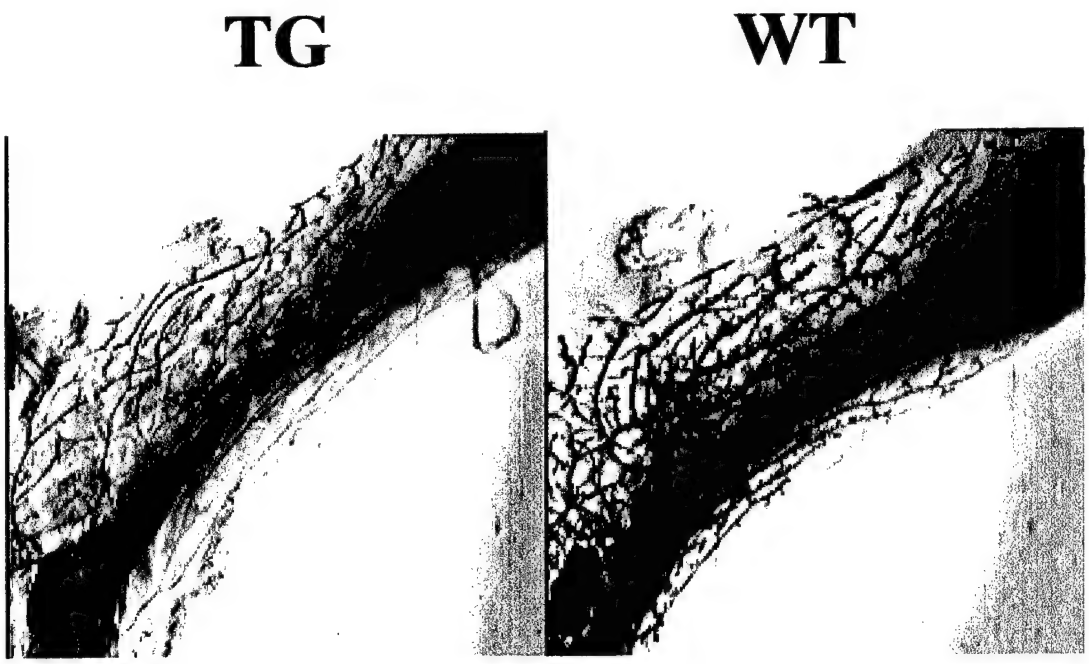
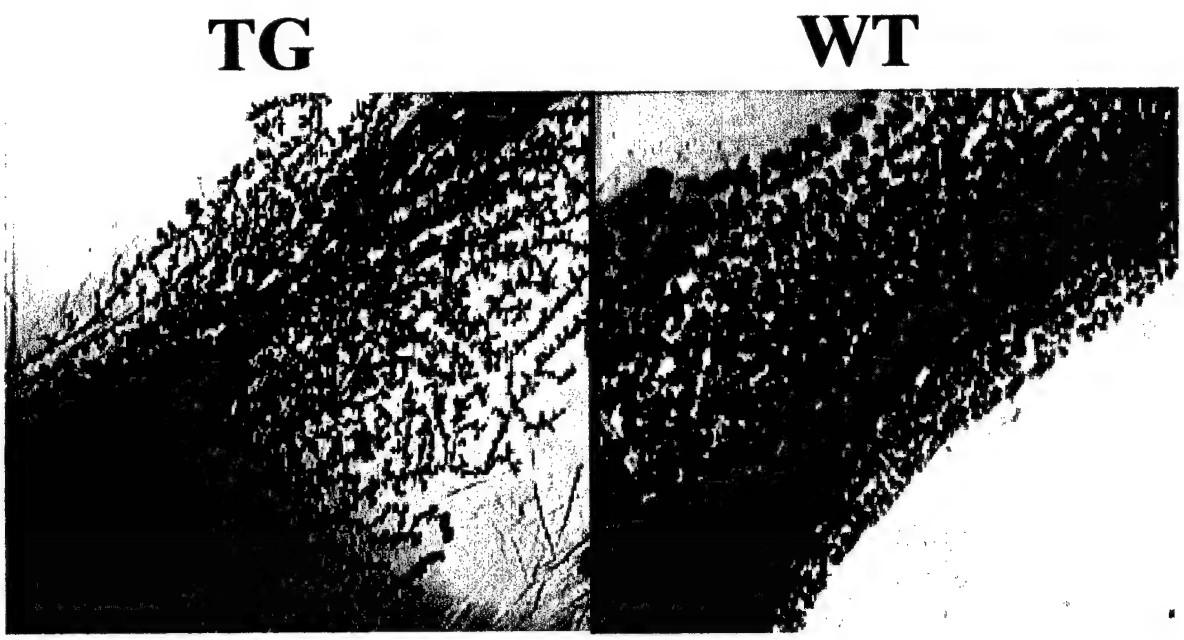


Figure 10: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 68 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

A.



B.

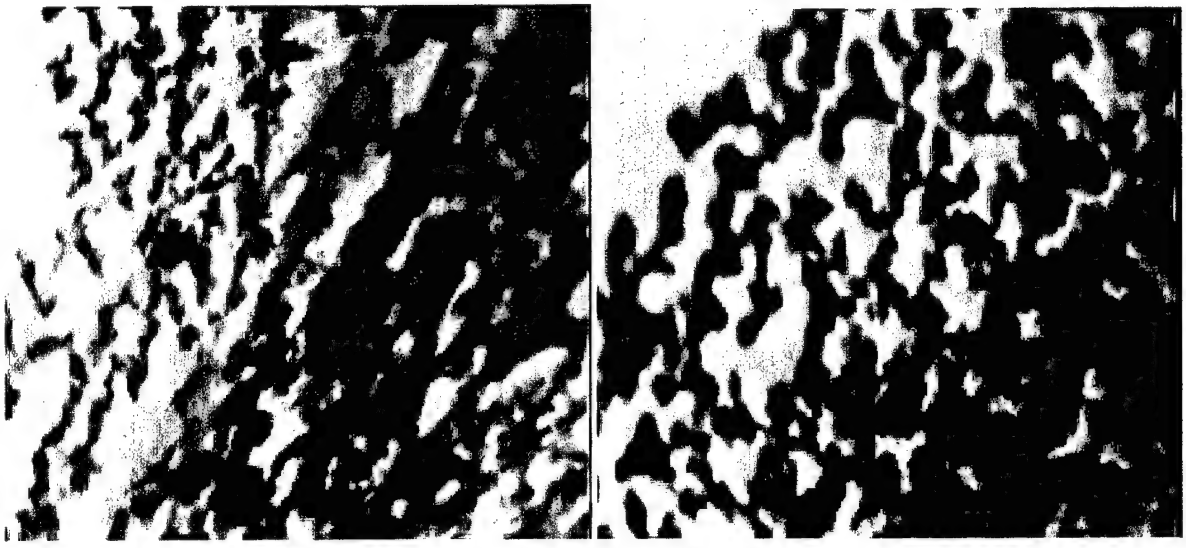
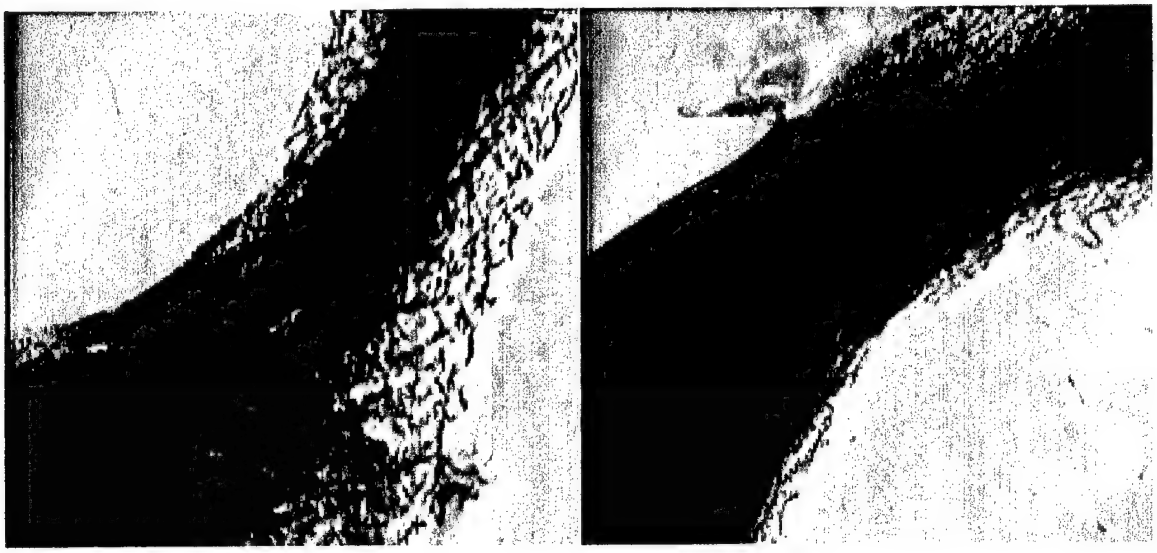


Figure 11: Overexpression of human E6-AP in mouse mammary gland has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

A.

TG WT



B.

Figure 12: Overexpression of human E6-AP in mouse mammary gland has no significant effect on 15 days involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

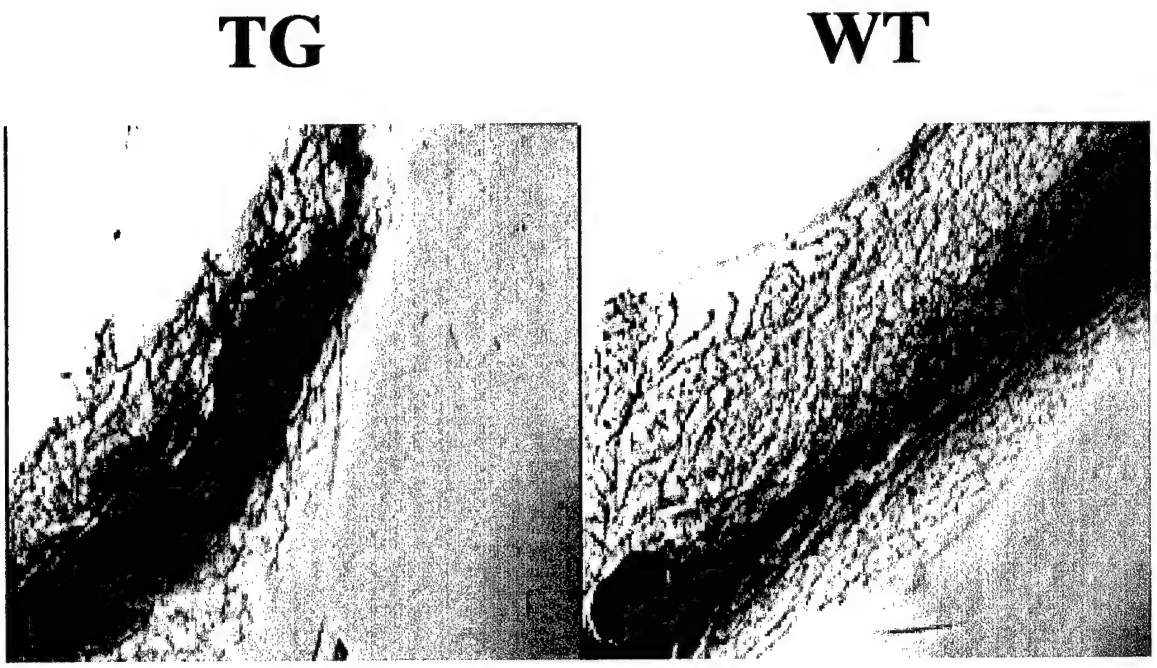


Figure 13: Overexpression of human E6-AP in mouse mammary gland results in smaller mammary glands 8 weeks after involution. Whole mount analyses of mammary glands from 8 weeks involuting mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

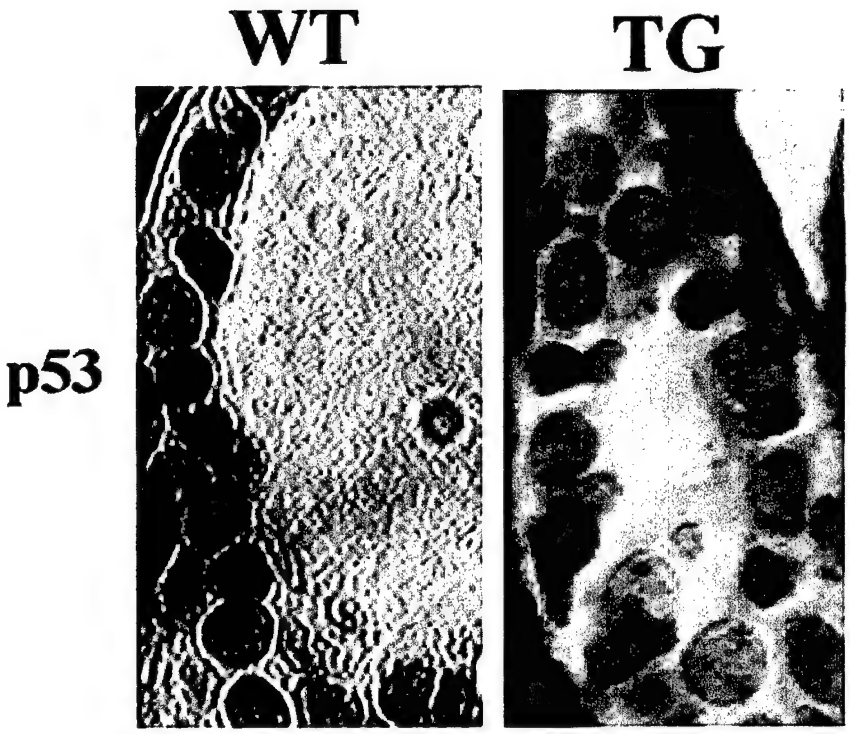


Figure 14: Expression analysis of p53 in normal and transgenic mammary glands by immunohistochemistry. The expression of p53 was analyzed by immunohistochemistry using an anti-p53 antibody. Positive signal for p53 is seen as (brown) spots. WT, Wild-type mammary gland; TG, Transgenic mammary gland.

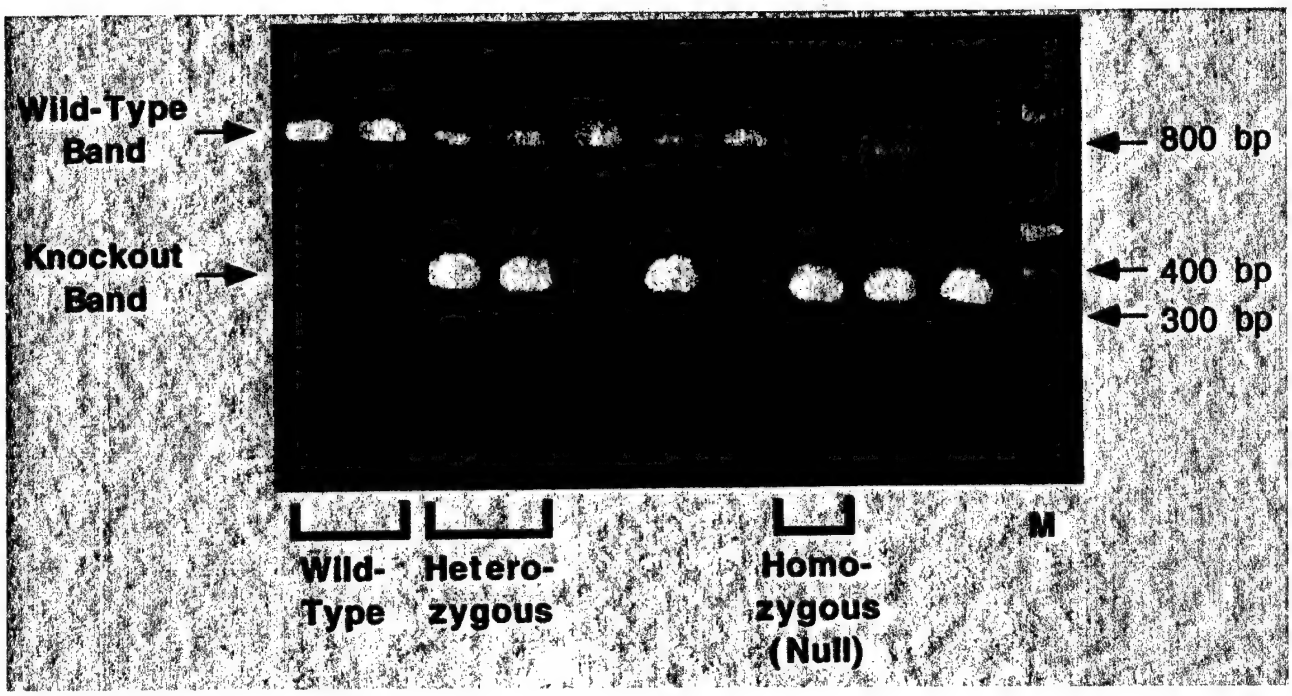


Figure 15: Screening of E6-AP KO (null) animals by PCR method. In order to identify E6-AP null mice, a PCR screening method was developed. To develop PCR screen 3 pairs of primer sets were designed. These primers, successfully amplify the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type only generate a 750 bp long band, whereas homozygous E6-AP null animals generate only 350 bp long fragment and heterozygous animals contain both bands.

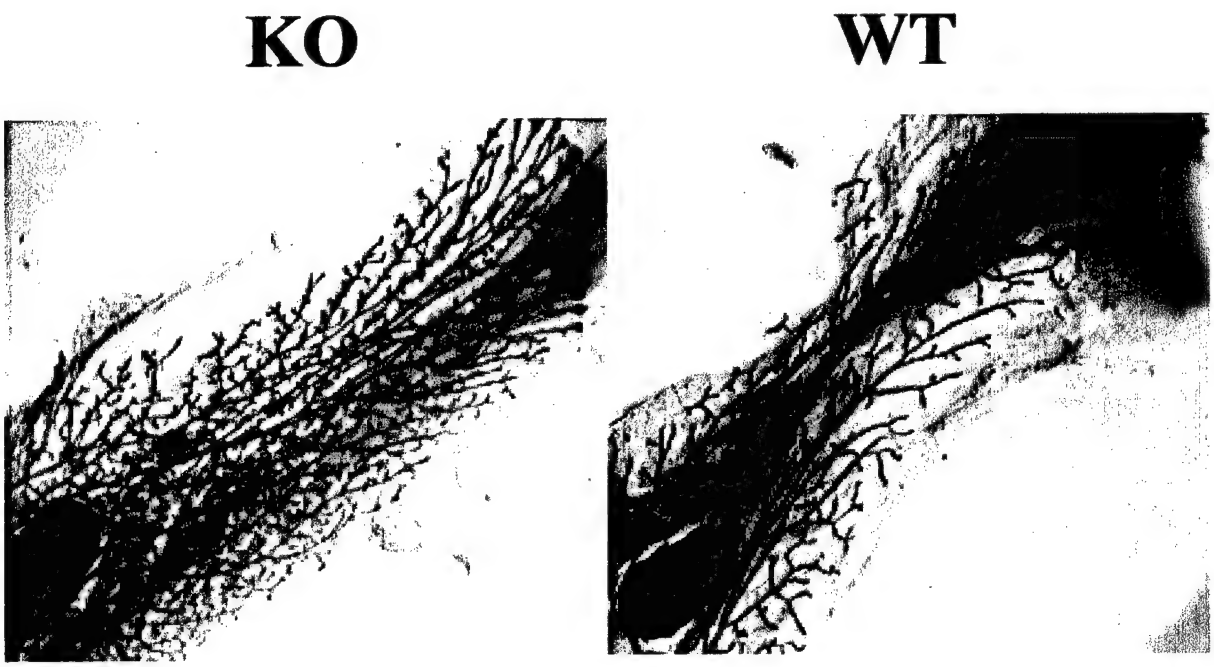


Figure 16: Loss of E6-AP expression results in increased alveolar buds. Whole mount analyses of mammary glands from 8 weeks old virgin mice were performed from wild-type (WT) and E6-AP knockout mice (KO).

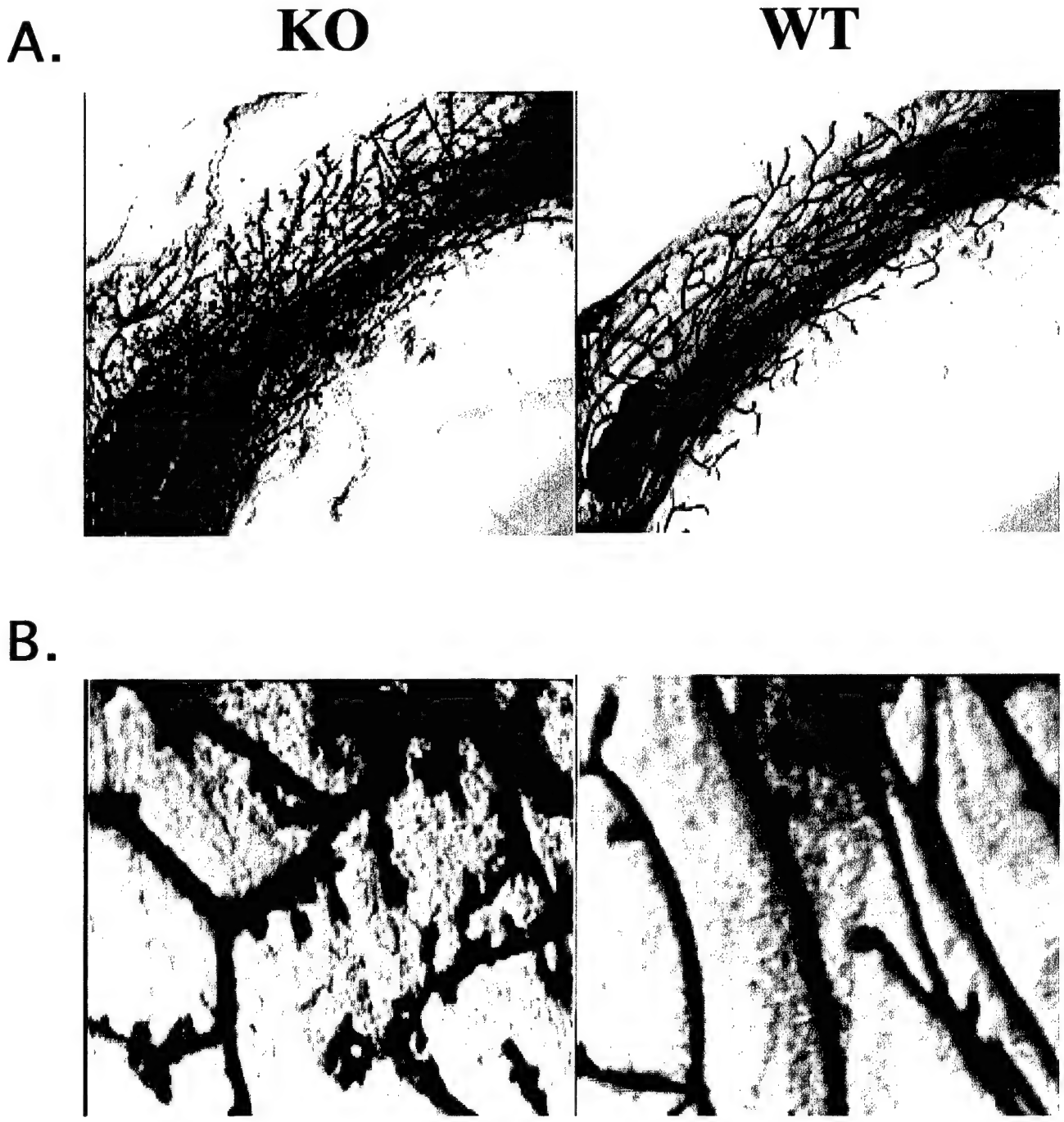


Figure 17: Loss of E6-AP expression results in increased alveolar buds. Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and E6-AP knockout mice (KO). (A) 5X (B) 20X

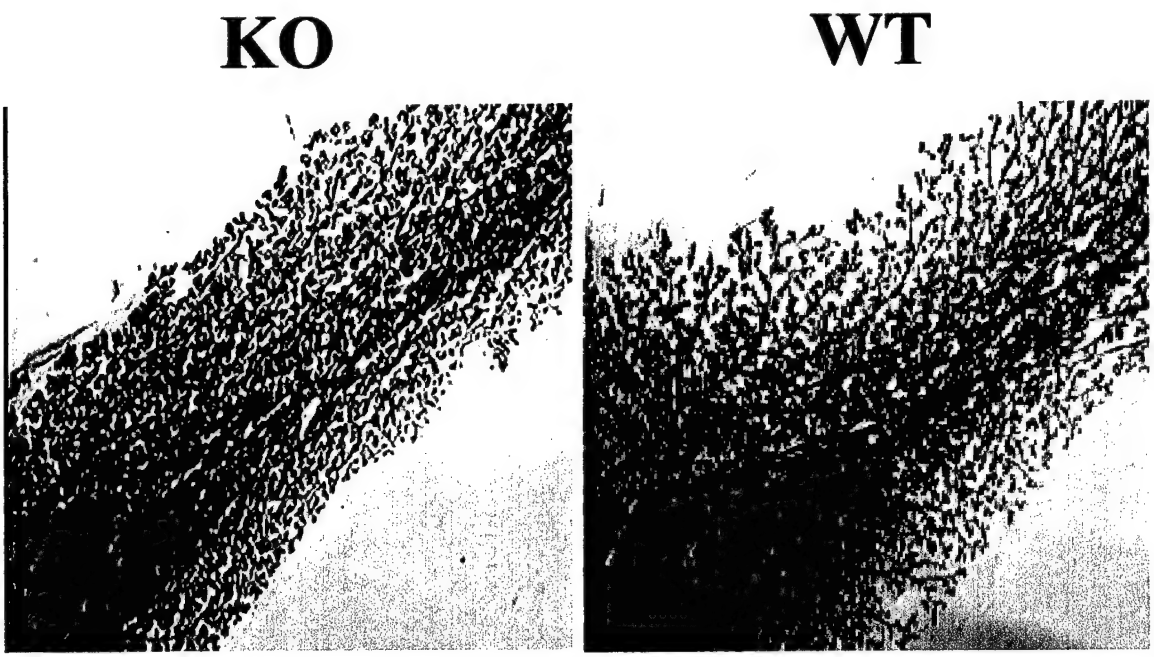


Figure 18: Loss of E6-AP expression has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO).

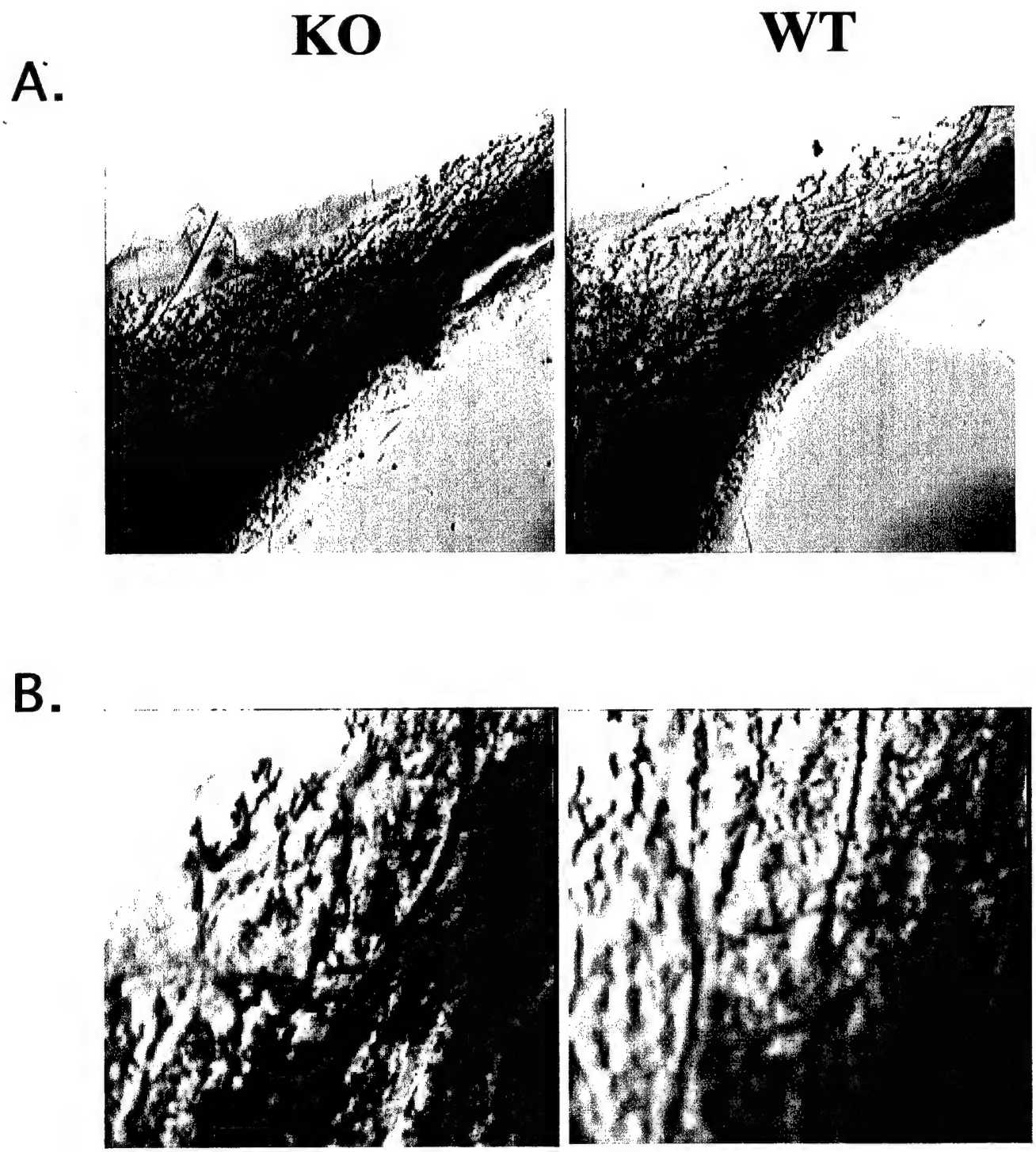


Figure 19: Loss of E6-AP expression has no significant effect on involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO). (A) 5X (B) 20X

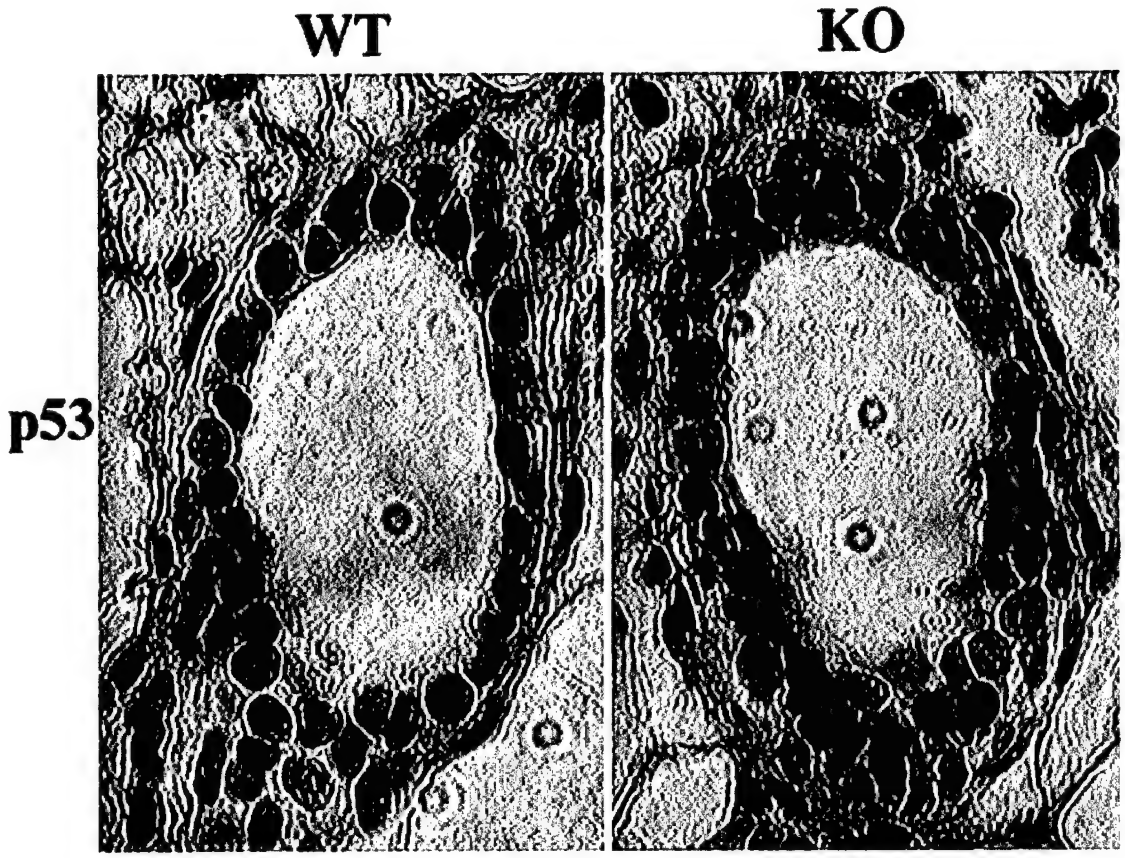


Figure 20: Expression analysis of p53 in normal and E6-AP knockout mammary glands by immunohistochemistry. The expression of p53 was analyzed by immunohistochemistry using an anti-p53 antibody. Positive signal for p53 is seen as (brown) spots. WT, Wild-type mammary gland; KO, E6-AP knockout mammary gland.

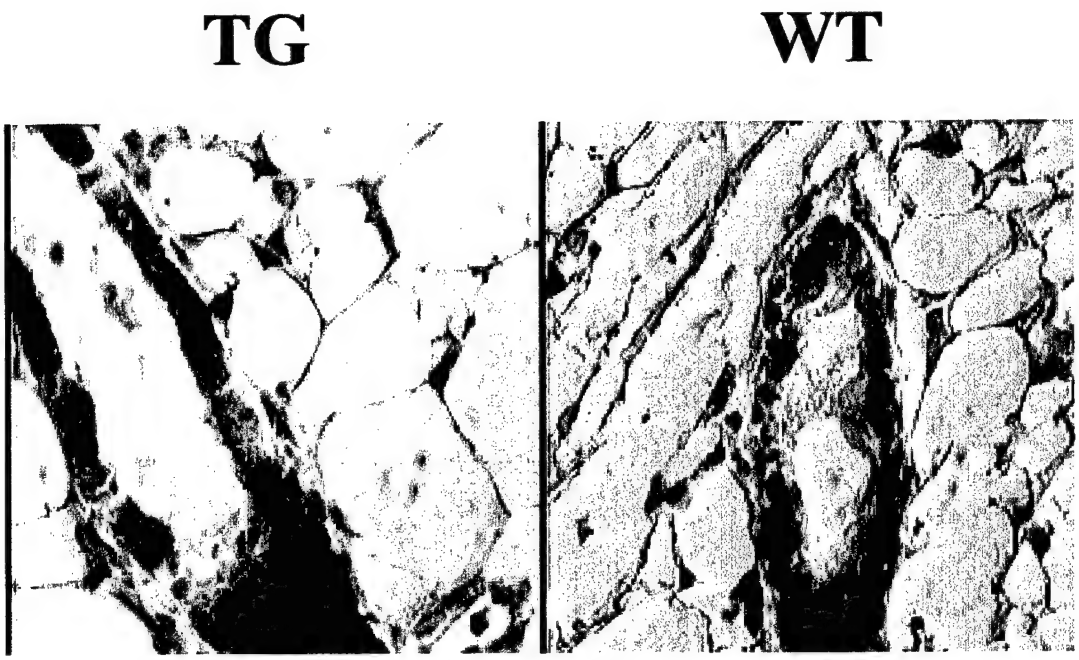


Figure 21: Expression analysis of transgene (MMTV-mutant E6-AP) in the mouse mammary glands. E6-AP expression in the mouse mammary glands was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal is seen as dark (brown) spots. WT, Wild-type mammary gland; TG, ligase-defective mutant E6-AP transgenic mammarygland.

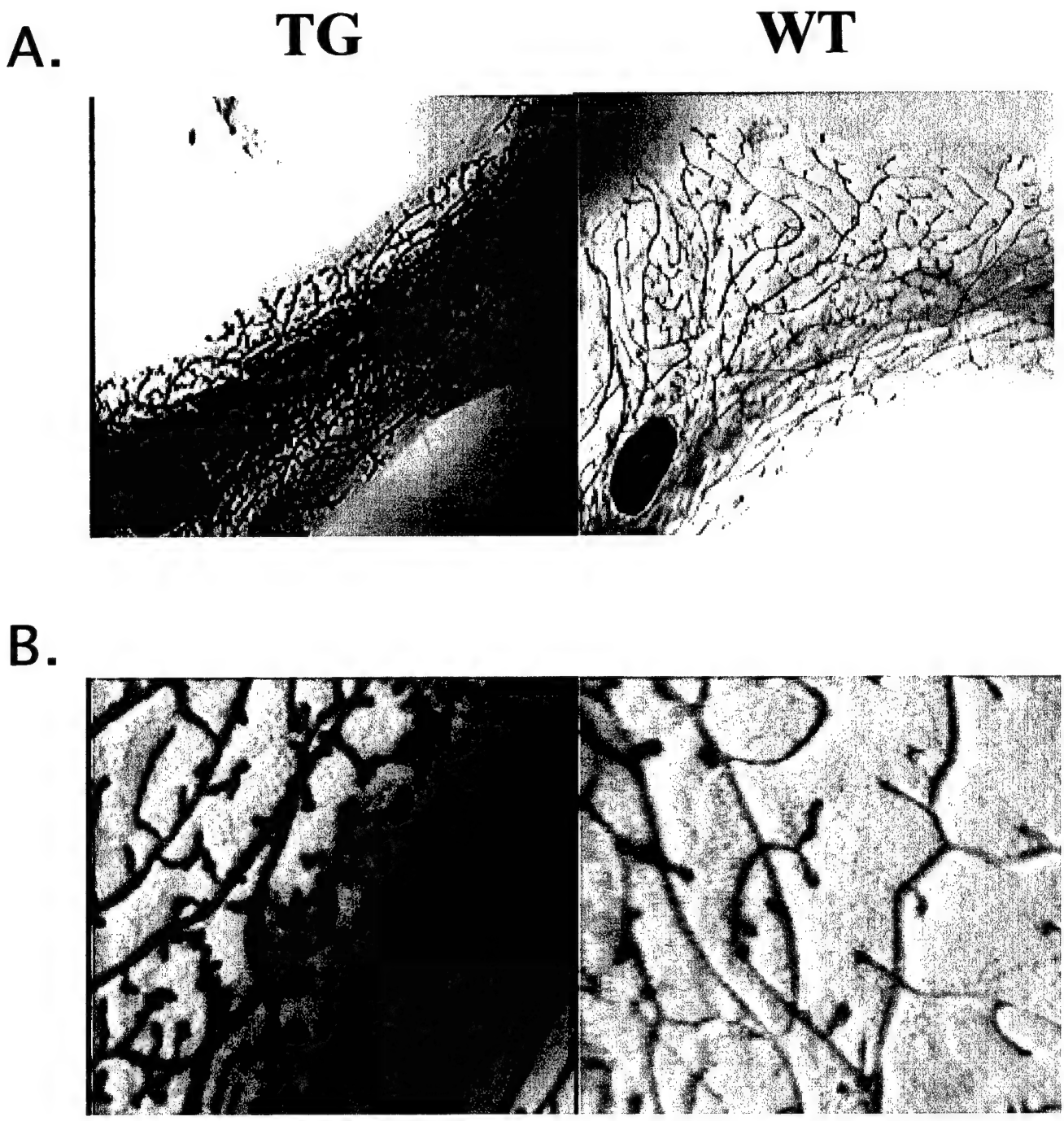


Figure 22: Overexpression of human ligase-defective mutant E6-AP in mouse mammary gland results in increased alveolar buds.
Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and E6-AP transgenic (TG). (A) 5X (B) 20X

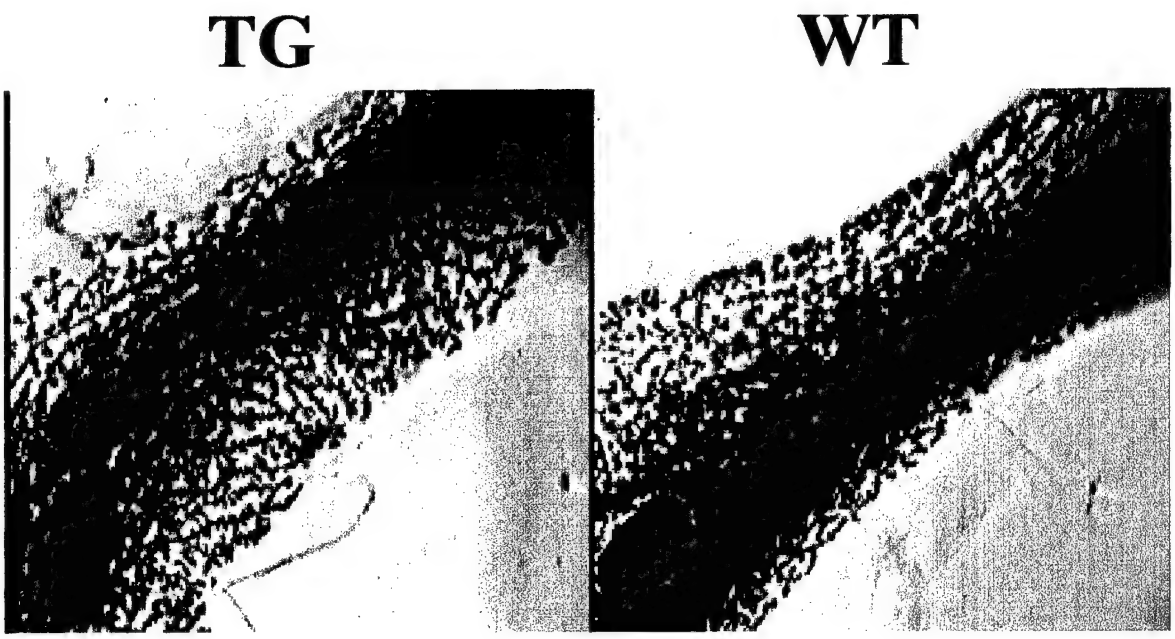


Figure 23: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG).

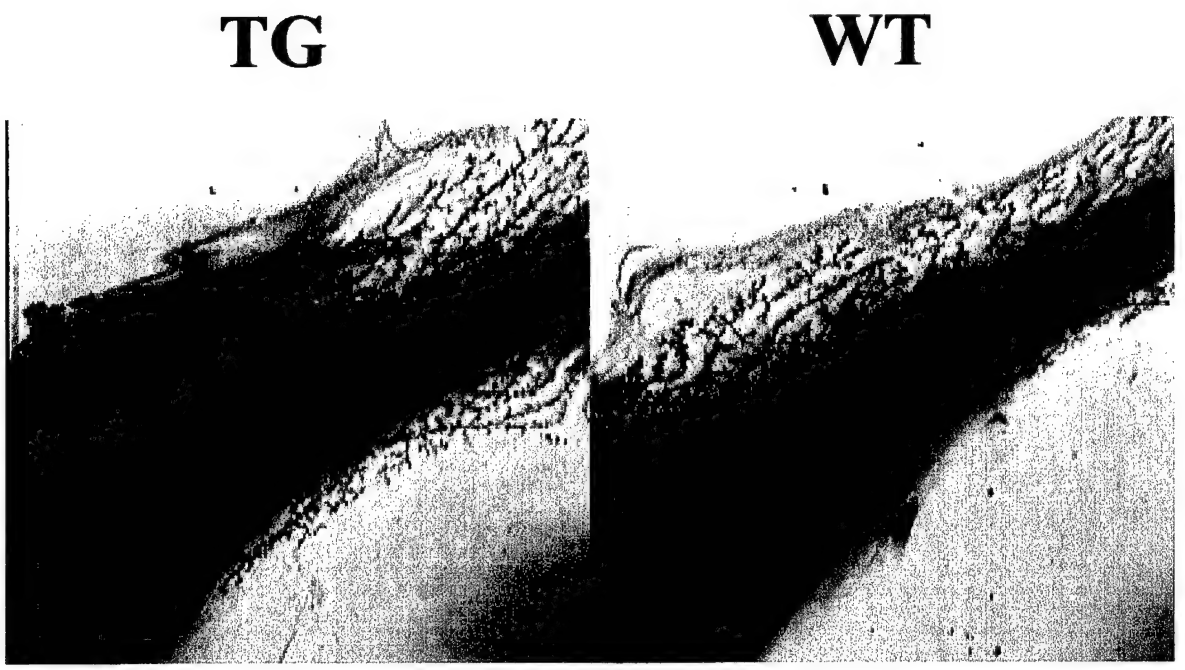


Figure 24: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland has no significant effect on 15 days involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG).

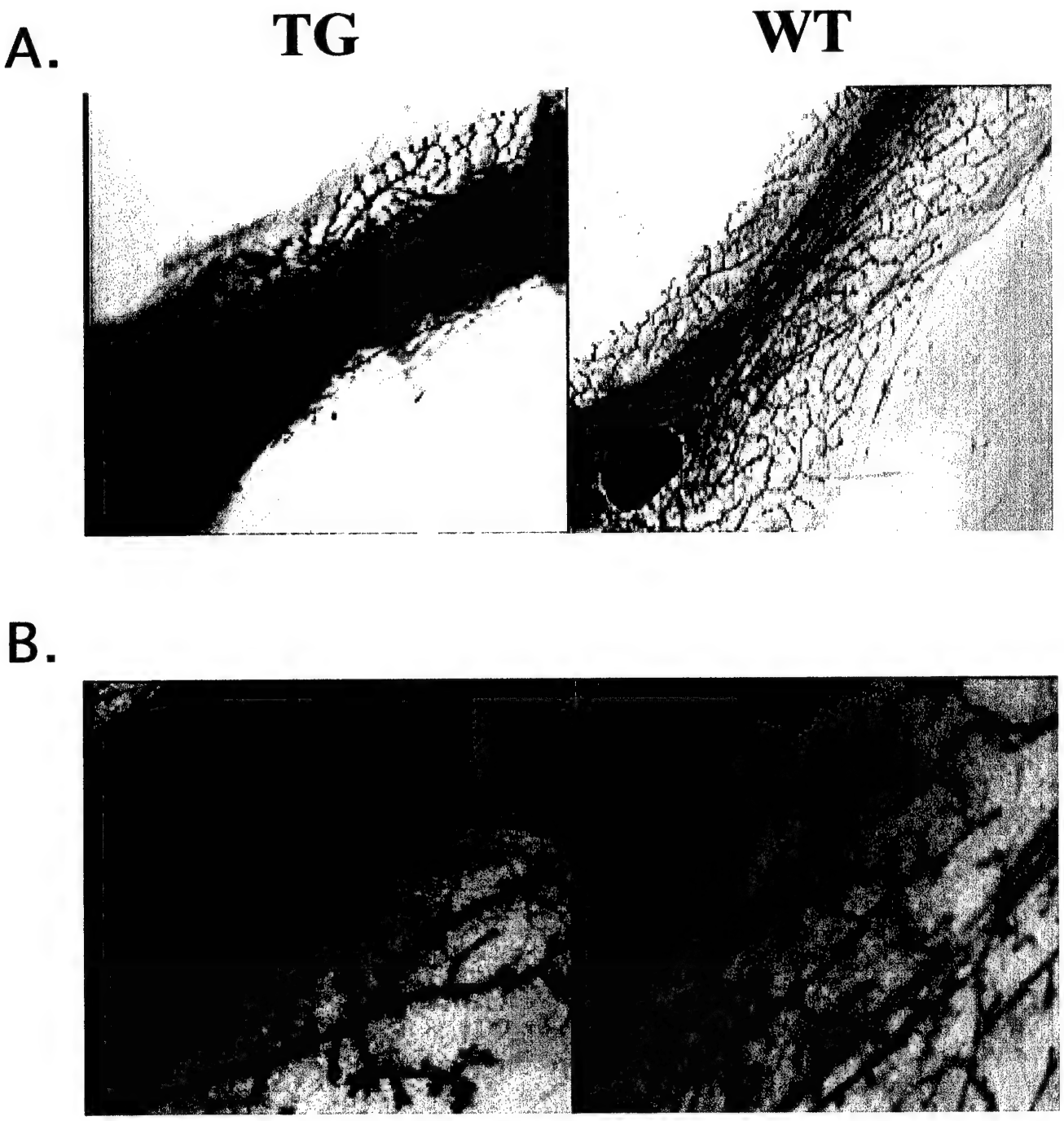


Figure 25: Overexpression of ligase-defective mutant E6-AP in mouse mammary gland results in increased alveolar buds 8 weeks after involution. Whole mount analyses of mammary glands from 8 weeks involuting mammary glands were performed from wild-type (WT) and MMTV-mutant E6-AP transgenic mice (TG). (A) 5X (B) 20X

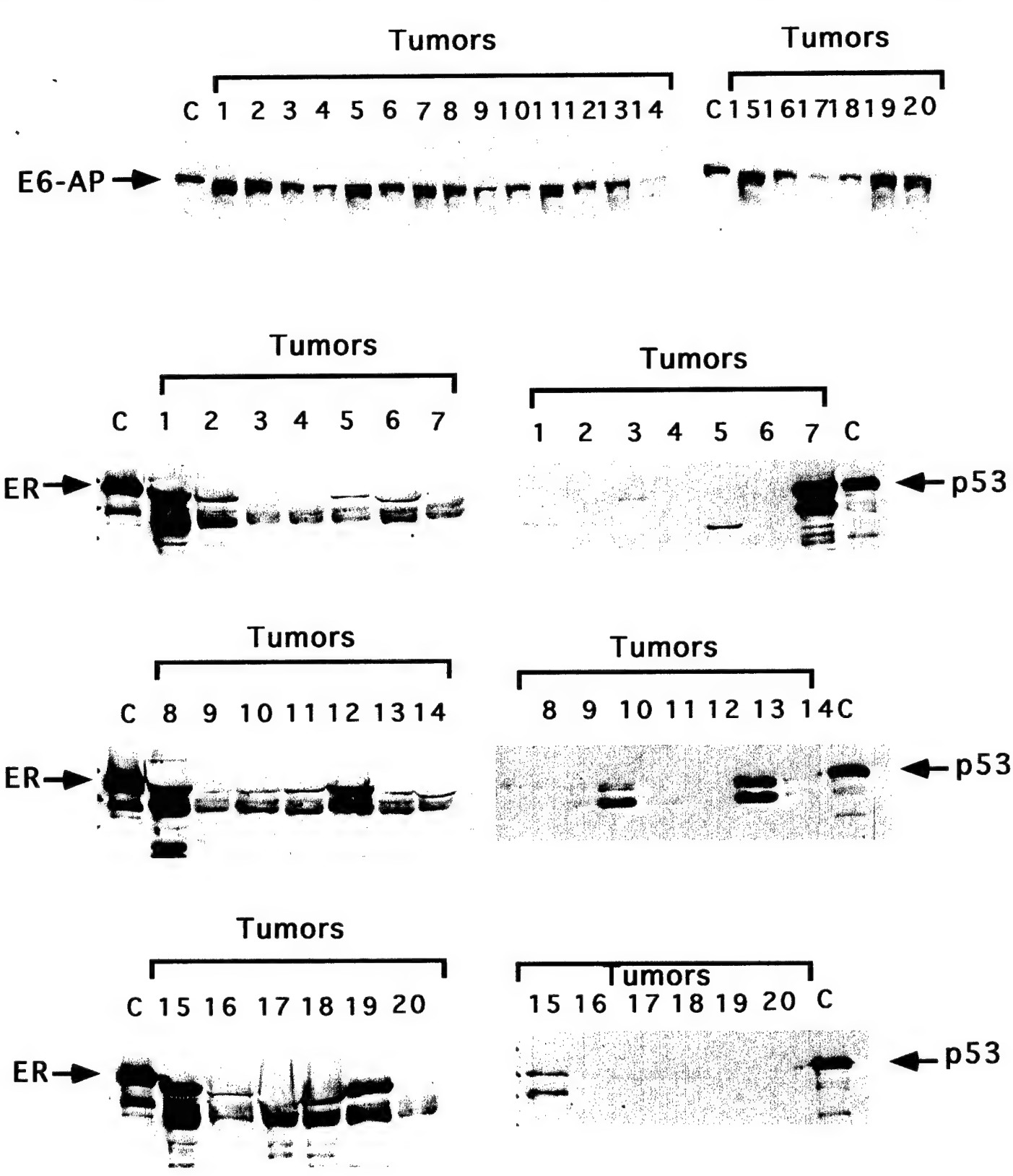


Figure 26: Expression analyses of E6-AP, ER and p53 in human biopsy tumor samples. Tumor samples were collected from the breast cancer center at the Baylor college of Medicine, Houston and expression of E6-AP, ER and p53 were determined by Western blot using E6-AP, ER and p53 specific antibodies.
C, control purified proteins.

Tumor #	E6-AP	ER	Tumor #	E6-AP	ER	Tumor #		ER
1	++++	++++	21	+	+	41	++	+
2	+++	+	22	+++	++	42	++	++++
3	++	-	23	++++	++++	43	+	++
4	+	-	24	+++	+++	44	+	++
5	++++	-/+	25	++	-	45	++	++
6	++	-/+	26	++	++++	46	+++	-/+
7	++	-	27	-/+	-/+	47	+++	-/+
8	+++	+++	28	++	-/+	48	+	-/+
9	+	-/+	29	++	-/+	49	++	-/+
10	++	-/+	30	++	+++	50	++++	+
11	+++	+	31	++++	++++	51	++++	+++
12	++	++	32	++	+	52	++++	+
13	++	-/+	33	+++	++	53	+++	++
14	-/+	-/+	34	+++	+	54	+++	++
15	++++	++++	35	++	+	55	++++	-/+
16	++	-/+	36	+++	-	56	+++	-/+
17	-/+	-	37	+++	++++			
18	+	+	38	+++	+			
19	++++	++	39	++++	+++			
20	++++	-	40	+	++			

Figure 27: Correlation of the expression of E6-AP with that of ER-alpha in breast tumors.
Expression levels of E6-AP and ER-alpha from western blot analysis was artificially graded according to the density of bands. "-" represents negative expression, whereas "-/+" represents very low expression. From "+" to "++++" represent the gradually increasing levels of expression from low to high. Spreaman Rank Correlation Coefficient for the expression of E6-AP with that of ER-alpha is 0.38, p=0.004.

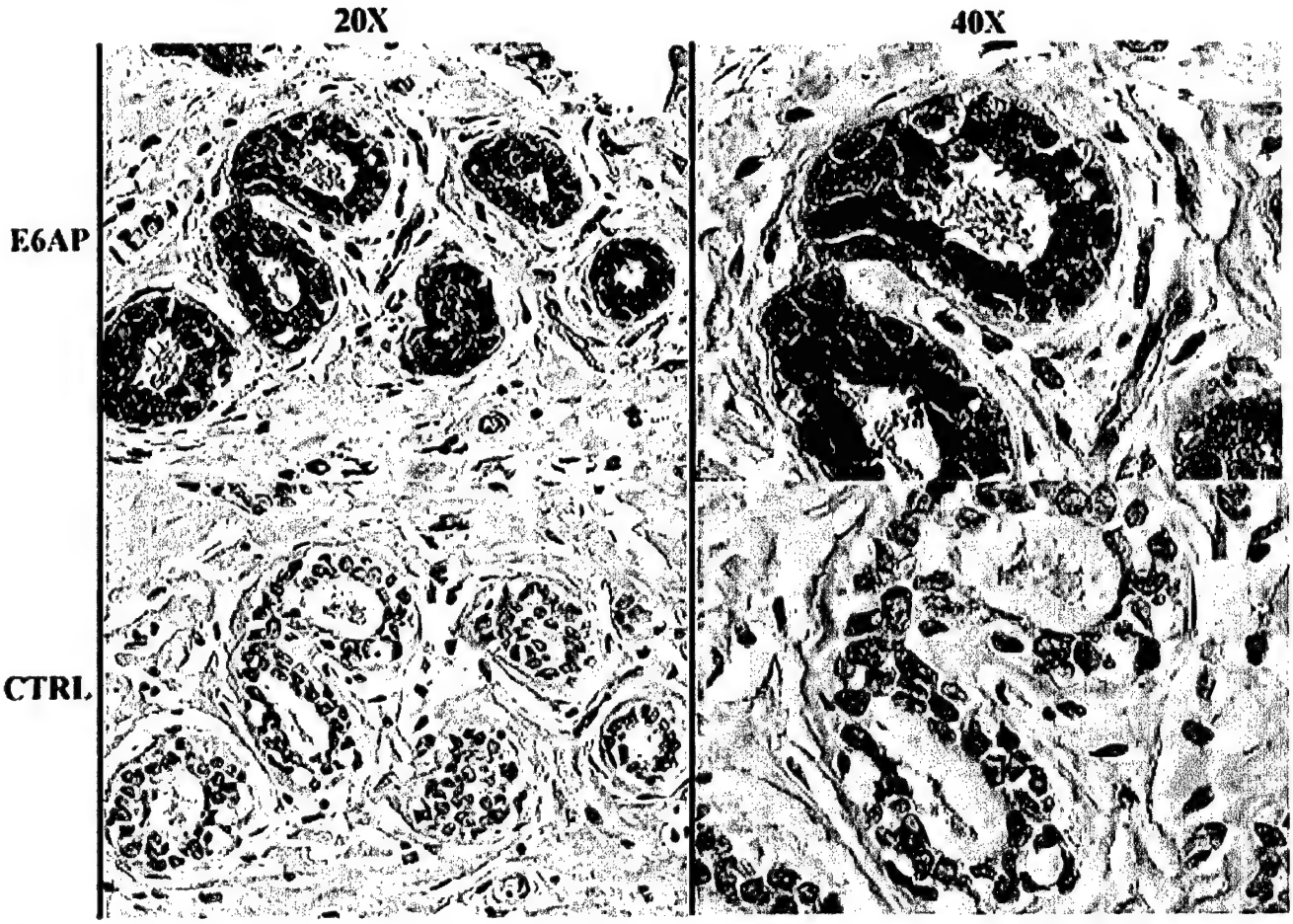


Figure 28: Expression analysis of E6-AP in normal human breast tissues by immunohistochemistry. The expression of endogenous E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. E6-AP, E6-AP polyclonal antibody; CTRL, no primary antibody.

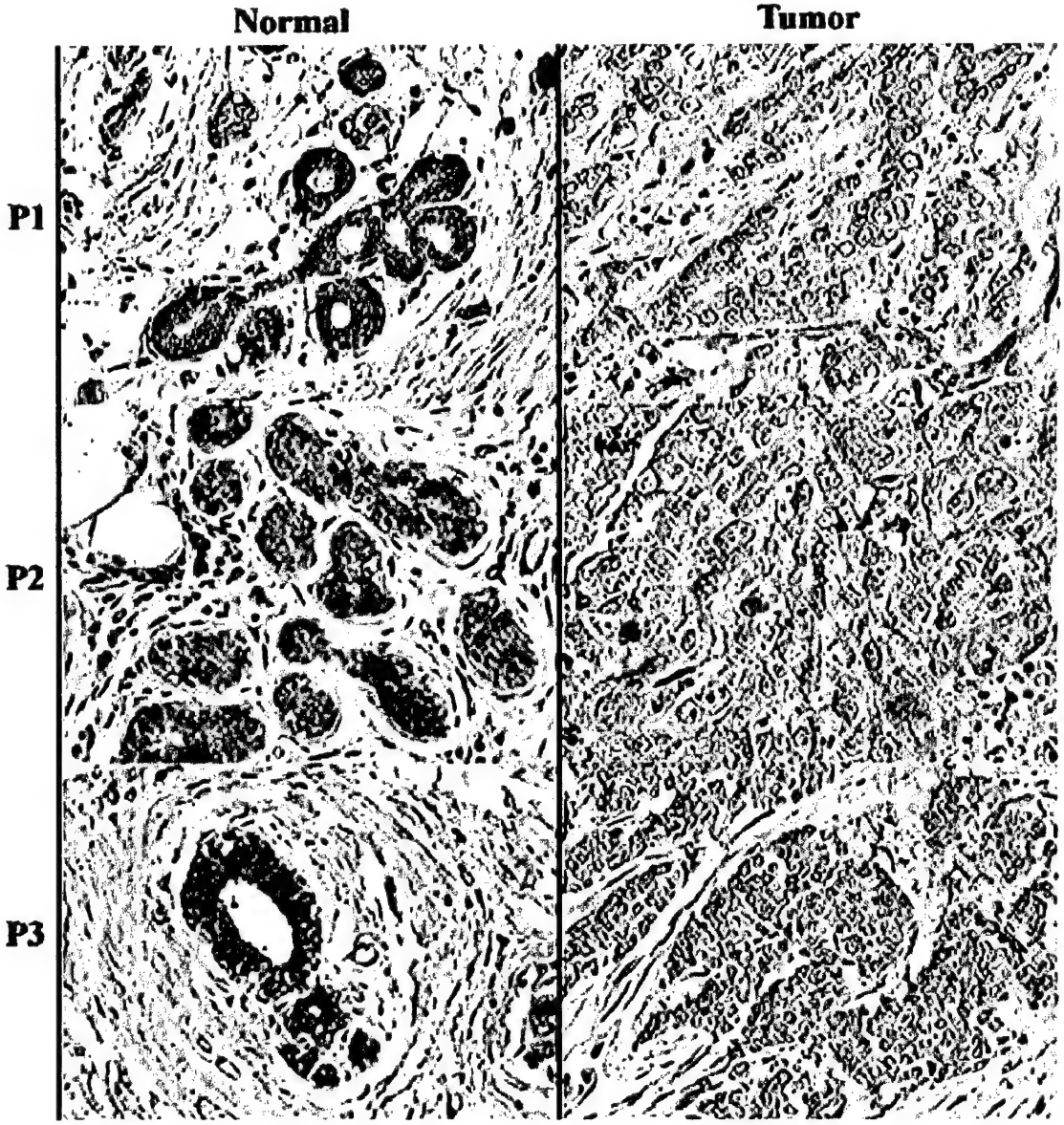


Figure 29: Expression analysis of E6-AP in human breast tumors and adjacent normal tissues by immunohistochemistry. The expression of E6-AP was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (brown) spots. P1, Patient1; P2, Patient 2; P3, Patient 3.

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
SI & SIIB	32	7, 2	<0.01*
SIIA & SIIB	52.5	7, 7	>0.05
SIIIA & SIIB	59.5	6, 1	<0.01*
SI & SIIA	115	9, 5	>0.1
SI & SIIIA	50.5	6, 3	>0.1

Figure 30: Comparison of the expression level of E6-AP between different stages of breast cancer. Expression levels of E6-AP were analyzed in human tumors of different stages by immunohistochemistry. Then the expression of E6-AP was compared within different stages by Wilcoxonrank-sum test. This analysis suggest that E6-AP expression is down in stage IIB with a p value of 0.01.

Appendix 2

2 Published Manuscripts

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Role of steroid receptor coactivators and corepressors of progesterone receptors in breast cancer

Xiuhua Gao and Zafar Nawaz

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Review

Progesterone receptors – animal models and cell signaling in breast cancer

Role of steroid receptor coactivators and corepressors of progesterone receptors in breast cancer

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Abstract

Progesterone, an ovarian steroid hormone, plays a key role in the development and function of the mammary gland, as it also does in the uterus and the ovary. The action of progesterone is mediated through its intracellular cognate receptor, the progesterone receptor (PR), which functions as a transcription factor that regulates gene expression. As with other nuclear receptors, coregulators (coactivators and corepressors) recruited by the liganded or unliganded PR, either to enhance or to suppress transcription activity, modulate the function of the PR. Mutation or aberrant expression of the coregulators might thus affect the normal function of the PR and hence disrupt the normal development of the mammary gland, which may lead to breast cancer.

Keywords: breast cancer, coactivator, corepressor, progesterone receptor

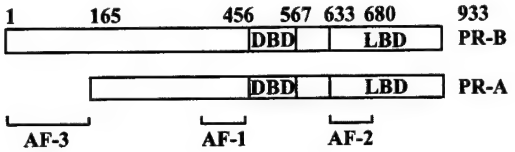
Introduction

The progesterone receptor (PR) is a member of the nuclear receptor superfamily, which specifically regulates the expression of target genes in response to the hormonal stimulus. In the absence of progesterone, the PR is sequestered in a nonproductive form associated with heat shock proteins and other cellular chaperones. In this state, the PR is considered unable to influence the rate of transcription of its cognate promoters [1]. On binding with progesterone, the PR undergoes a series of events, including conformational changes, dissociation from heat shock protein complexes, dimerization, phosphorylation, and nuclear translocation, which enables its binding to progesterone-response elements within the regulatory regions of target genes. The binding of PR to the progesterone-response elements is followed by the recruitment of coactivators and the basal transcription machinery, leading to the upregulation of target gene transcription.

The PR exists as two isoforms in most rodents and humans, PR-A and PR-B, which are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters [2]. The difference between PR-A and PR-B is that PR-A is a truncated form of PR-B. In humans, the N-terminal 164 amino acids of PR-B are missing in isoform PR-A. Detailed molecular dissection has identified two distinct activation function domains (AFs) within both PRs: AF-1, which is located in the N-terminal region, is ligand independent; AF-2, which is ligand dependent, is contained in the ligand-binding domain that is located in the C-terminal region. A DNA-binding domain and the hinge region are

AF = activation function domain; CBP = CREB-binding protein; E6-AP = E6-associated protein; ER = estrogen receptor; N-CoR = nuclear receptor corepressor; PR = progesterone receptor; RPF1 = receptor potentiation factor-1; RTA = repressor of tamoxifen transcriptional activity; SMRT = silencing mediator of retinoid and thyroid receptor; SRA = steroid receptor RNA activator; SRC = steroid receptor coactivator; Uba3 = ubiquitin-activating enzyme.

Figure 1



Schematic representation of the progesterone receptor PR-A and PR-B proteins. The DNA-binding domain (DBD), the ligand-binding domain (LBD) and activation function domains (AFs) are indicated.

mapped to the central region of both receptors. Furthermore, a unique activation function domain, AF-3, is contained in the upstream segment of PR-B that is missing in PR-A (Fig. 1).

Progesterone and estrogen are essential regulators of female reproductive activity. Through their cognate receptors, estrogen and progesterone regulate the normal development of the ovary, the uterus and the mammary gland, and play key roles in the tumorigenesis of these tissues. It has been demonstrated by estrogen receptor (ER) and PR knockout mice that estrogen controls the early ductal morphogenesis of the mammary gland, whereas progesterone controls ductal branching and alveolar development of the mammary gland during pregnancy [3].

Although the two forms of the PR have similar structures and are identical in DNA and ligand binding, *in vitro* studies using a reconstituted progesterone-responsive transcription system in mammalian cells revealed that PR-A and PR-B are not functionally identical. In most cases, PR-B acts as a potent activator of transcription of target genes, whereas PR-A acts as a dominant repressor of transcription of PR-B as well as a few other nuclear receptors [4]. The AF-3 domain in PR-B is partially responsible for the higher transcriptional activity of PR-B relative to PR-A. Moreover, an inhibitory function domain that is located in the N-terminus of both receptor isoforms has been identified [2]. This inhibition function domain can inhibit the activity of AF-1 and AF-2 but not that of AF-3, which explains why PR-B is a potent activator of transcription. The inhibition function domain is functionally independent and is transferable; when placed upstream of the ER, the inhibition function domain can also suppress ER activity.

The relative expression of PR-A and PR-B in the target tissues is dependent on species, cellular context, and the physiological and hormonal status. The ratio of PR-A to PR-B in specific tissues or cell types defines the physiological and pharmacological responses to progesterone. In the mammary gland, the ratios of PR-A to PR-B are constant from puberty to pregnancy, although there are species differences [5]. From PR-A knockout mice, it has been demonstrated that PR-B is mainly responsible for the normal proliferative and differentiative responses of the mammary gland to progesterone, because PR-A knockout mice exhibit a similar phenotype to PR knockout mice [3]. Overexpression of PR-A over PR-B in transgenic mice results in extensive epithelial cell hyperplasia, in excessive ductal branching, and in a disorganized basement membrane. All these features are associated with neoplasia [6]. Furthermore, the development of the mammary gland in PR-B overexpressed transgenic mice is also abnormal [7], indicating that a regulated expression of PR-A and PR-B and the native ratios of the two isoforms are critical to the appropriate responsiveness of the mammary gland to progesterone. Consistent with the findings from animal studies, very low levels of PR-B and a consequently high PR-A:PR-B ratio were found in a significant proportion of human breast cancer samples [8]. Taking these findings together, it can be inferred that imbalance of PR-A versus PR-B may be associated with the development, progression or prognosis of breast cancer.

Coactivators are factors that can interact with nuclear receptors in a ligand-dependent manner and enhance their transcriptional activity. Corepressors are factors that interact with nuclear receptors and repress their transcriptional activity. Both types of coregulators are required for efficient modulation of target gene transcription by the PR [9]. Changes in the expression level and pattern of PR coactivators or corepressors, or mutation of their function domains, might therefore affect the transcriptional activity of the PR and hence cause disorders of its target tissues, including the mammary gland. The present review will describe the coactivators and corepressors that are involved in the transcriptional modulation of PRs, with emphasis on their roles in breast cancer development and progression.

Progesterone receptor coactivators

The steroid receptor coactivator family

The steroid receptor coactivator (SRC) family is composed of three distinct but structurally and functionally related members: SRC-1 (nuclear receptor coactivator 1), SRC-2 (transcription intermediary factor 2/glucocorticoid receptor-interacting protein 1/nuclear receptor coactivator 2), and SRC-3 (p300/CREB-binding protein [CBP] cointegrator-associated protein/receptor-associated coactivator 3/activator of thyroid and retinoid receptors/amplified in breast cancer 1/thyroid receptor activator molecule 1). SRC-1 was the first identified coactivator for the steroid receptor superfamily, which was cloned and characterized in 1995 [10]. SRC-2 and SRC-3 were then identified thereafter by several laboratories [9].

Sequence analysis of SRC proteins identified a basic helix–loop–helix domain and two Per–Arnt–Sim domains in the amino-terminal region. The basic helix–loop–helix/

Per–Arnt–Sim domain is highly conserved among the SRC members, and it serves as a DNA binding and protein dimerization motif in many transcription factors [11]. Following the basic helix–loop–helix/Per–Arnt–Sim domain, there are a centrally located receptor-interacting domain and a C-terminal transcriptional activation domain. Detailed analysis revealed three conserved LXXLL motifs (nuclear receptor box) in the receptor-interacting domain, which appear to contribute to the specificity of coactivator–receptor interaction. Histone acetyltransferase activity was identified in the C-terminal region of SRC members, and there also exist activation domains that can interact with the CBP.

All three members of the SRC family interact with the PR and enhance its transcriptional activation in a ligand-dependent manner [12,13]. Targeted deletion of the SRC-1 gene in mice has indicated that SRC-1 is important for the biological actions of progesterone in mammary gland development since the hormone-induced ductal elongation and alveolar development is greatly impaired in the null mice [14]. In the meantime, the expression of SRC-2 mRNA was elevated in SRC-1 null mice, suggesting that SRC-2 can partially compensate for SRC-1 function [14].

SRC-3 is the most distinct among the three members. It coactivates not only the nuclear receptors, but also other unrelated transcription factors such as those in the cAMP or cytokine pathways [15]. Compared with the widespread expression of SRC-1 and SRC-2, expression of SRC-3 is restricted to the mammary gland and several other tissues [16]. Disruption of the SRC-3 gene in mice causes severe growth and reproductive defects, including the retardation of mammary gland development [17]. Furthermore, amplification and overexpression of SRC-3 were observed in 10% and 64% of human primary breast cancers, respectively [18]. This observation indicates that SRC-3 is not only essential for the normal mammary development, but also plays a role in breast tumorigenesis.

E6-associated protein/RPF1

E6-associated protein (E6-AP) and RPF1, the human homolog of yeast RSP5, are E3 ubiquitin-protein ligases that target proteins for degradation by the ubiquitin pathway. They are also characterized as coactivators of steroid receptors. It has been demonstrated by transient transfection assay that RPF1 and E6-AP could potentiate the ligand-dependent transcriptional activity of the PR, the glucocorticoid receptor, and other nuclear receptors [19,20]. Furthermore, RPF1 and E6-AP acted synergistically to enhance PR transactivation [20]. Additionally, the coactivation functions of E6-AP and RPF1 are separable from the E3 ubiquitin-protein ligase activity, as ubiquitin ligase-defective E6-AP and RPF1 exhibited normal coactivation function.

E6-AP is expressed in many tissues, including the mammary gland. From its ability to coactivate the PR and the ER in a hormone-dependent manner, it was assumed that E6-AP is an essential regulator for the development of normal mammary gland and mammary tumors. The first evidence of a relationship between E6-AP and breast cancers was obtained from the study of a spontaneous mouse mammary tumorigenesis model, which demonstrated that E6-AP was overexpressed in tumors when compared with normal tissues [21].

We recently examined the expression pattern of E6-AP in biopsy samples of human breast cancers, and our results showed that E6-AP expression was decreased in tumors in comparison with the adjacent normal tissues (Gao *et al.*, unpublished data, 2002). Furthermore, we demonstrated that the decreased expression of E6-AP was stage dependent, and that the expression of E6-AP was inversely correlated with that of the ER in breast tumors. Since the ER plays a major role in breast cancer development and the PR is a target of estrogen, the changes of the expression level of E6-AP might interfere with the normal functioning of the ER and the PR. Hence, E6-AP may participate in the formation and progression of breast tumors.

Steroid receptor RNA activator

The growing family of nuclear receptor coactivators has recently acquired a unique member, steroid receptor RNA activator (SRA) [22]. Differing from the other coactivators, SRA functions as a RNA transcript instead of as a protein. SRA specifically coactivates the transcriptional activity of steroid receptors, including the PR, the ER, the glucocorticoid receptor, and the androgen receptor. It has been demonstrated that SRA exists in a ribonucleoprotein complex containing SRC-1 and that it mediates transactivation through the AF-1 domain located at the N-terminal region of nuclear receptors, distinguishing it from the other coactivators.

SRA is expressed in normal and malignant human mammary tissues [23,24]. An elevated expression of SRA was found in tumors compared with the adjacent normal region [24]. Although it is currently unknown whether the expression of SRA is correlated with that of the PR or the ER, the increase in the SRA levels in tumor cells may contribute to the altered ER/PR action that is known to occur during breast tumorigenesis.

L7/SPA

L7/SPA is a 27 kDa protein containing a basic leucine zipper domain. L7/SPA is an antagonist-specific transcriptional coactivator because it can only potentiate the partial agonist activity of some antagonists, including tamoxifen and RU486, but has no effect on the agonist-mediated transcription [25]. This unique property of L7/SPA suggests that it might play a role in the development of resistance to hormone therapy for breast cancers.

CBP/p300

CBP was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein, and p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties: both of them function as coactivators for multiple nuclear receptors as well as p53 and nuclear factor-κB [9], both possess intrinsic histone acetyltransferase activity, and both can recruit histone acetyltransferase and CBP/p300-associated factor [26]. Besides, CBP/p300 interacts with members of the SRC family and synergizes with SRC-1 in the transactivation of the ER and the PR [27].

Other coactivators

In addition to the coactivators already discussed, there are a few other proteins that have been demonstrated to upregulate the transcriptional activity of the PR. Chromatin high-mobility group protein 1, chromatin high-mobility group protein 2, TIP60 (Tat-interacting protein), proline-rich nuclear receptor coregulatory protein 1, proline-rich nuclear receptor coregulatory protein 2, Cdc25B, and GT198 all function as PR coactivators, as demonstrated by transient transfection assays [28–32]. Cdc25B is prominent among these coactivators in terms of its roles in breast cancer development, because Cdc25B transgenic mice exhibit mammary gland hyperplasia and increased steroid hormone responsiveness [31]. The significance of all these coactivators *in vivo* needs to be further investigated.

Progesterone receptor corepressors

Nuclear receptor corepressor/silencing mediator of retinoid and thyroid receptors

Nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptor (SMRT) are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with the nuclear receptors through the receptor-interacting domains located in the C-terminal portion of the proteins, while their transcriptional repression domains are mapped to the N-termini [33]. N-CoR and SMRT also associate with HDAC3 in large protein complexes, which is an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the nuclear receptors either in the absence of agonists (in the case of the thyroid receptor and the retinoid acid receptor) or in the presence of antagonists (in the case of steroid receptors) [33]. Since N-CoR and SMRT are common corepressors for transcription factors, slight alteration of their expression level in certain tissues might result in significant transcriptional changes, leading to altered development of the mammary gland, even tumors.

BRCA1

BRCA1 is a breast cancer susceptibility gene, and its inherited mutations are correlated with an increased risk of breast cancer and ovarian cancer [34]. The role of *BRCA1* in cancer development is unclear. In addition to its ability to coactivate p53 and to modulate p300/CBP expression, *BRCA1* is also a ligand-independent corepressor for the ER, the androgen receptor and the PR [35]. If *BRCA1* is mutated, all of these pathways will be more or less impaired. The effect of *BRCA1* in cancer development might therefore be multiplex.

Other corepressors

Ubiquitin-activating enzyme 3 (Uba3) is the catalytic subunit of the activating enzyme in the ubiquitin-like NEDD8 (neural precursor cell-expressed developmentally downregulated) conjugation (neddylation) pathway. Uba3 was recently demonstrated as a corepressor of the ER, the androgen receptor and the PR in mammalian transfection assays [36]. Uba3 inhibited the transactivation of the ER in a time-dependent manner, and neddylation activity of Uba3 is required for this suppression. This suggests that Uba3 suppresses steroid receptor activity by promoting the termination of receptor-mediated gene transcription rather than by interfering with the initial events.

Repressor of tamoxifen transcriptional activity (RTA) has recently been defined as a potent repressor of tamoxifen-mediated ERα transcriptional activity as well as an agonist of the ERβ, the glucocorticoid receptor, and the PR [37]. The interaction of RTA with the nuclear receptors requires the participation of RNA, because mutation of the RNA recognition motif in RTA compromises its ability to repress transcription [37].

The roles of Uba3 and RTA in mammary gland development and tumorigenesis await further study.

Summary

As a transcription factor, the PR activates target gene transcription in response to the hormonal stimulus, and its functions are modulated by coactivators and corepressors. Different coregulators exert their actions through different mechanisms, and involvement in the development of normal mammary gland and the formation or progression of tumors has been reported in some coactivators and some corepressors. The coactivators and corepressors of the PR so far identified are not PR specific, since they can also modulate the transactivation of many other nuclear receptors. In addition, no unique coregulators of PR-A or PR-B have been identified. Identification of PR-specific coregulatory proteins, especially PR-A interacting factors or PR-B interacting factors, is an important goal of future study.

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Review

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The roles of sex steroid receptor coregulators in cancer

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Keywords: nuclear receptors, steroid receptors, coregulators, coactivators, corepressors, cancer

Abstract

Sex steroid hormones, estrogen, progesterone and androgen, play pivotal roles in sex differentiation and development, and in reproductive functions and sexual behavior. Studies have shown that sex steroid hormones are the key regulators in the development and progression of endocrine-related cancers, especially the cancers of the reproductive tissues. The actions of estrogen, progesterone and androgen are mediated through their cognate intracellular receptor proteins, the estrogen receptors (ER), the progesterone receptors (PR) and the androgen receptor (AR), respectively. These receptors are members of the nuclear receptor (NR) superfamily, which function as transcription factors that regulate their target gene expression. Proper functioning of these steroid receptors maintains the normal responsiveness of the target tissues to the stimulations of the steroid hormones. This permits the normal development and function of reproductive tissues. It can be inferred that factors influencing the expression or function of steroid receptors will interfere with the normal development and function of the target tissues, and may induce pathological conditions, including cancers. In addition to the direct contact with the basal transcription machinery, nuclear receptors enhance or suppress transcription by recruiting an array of coactivators and corepressors, collectively named coregulators. Therefore, the mutation or aberrant expression of sex steroid receptor coregulators will affect the normal function of the sex steroid receptors and hence may participate in the development and progression of the cancers.

Introduction

The mammary gland, the ovary and the uterus in females, and the testis and the prostate gland in males are the main target tissues of sex steroid hormones including estrogen, progesterone, and androgen. Estrogen is important for the growth, differentiation and function of both female and male reproductive tissues [1,2], whereas progesterone is an essential regulator of the reproductive events associated with the establishment and maintenance of pregnancy, including ovulation, uterine and mammary gland development [3]. Androgen is involved in the development and physiological function of male accessory sex organs [4], and it is also indispensable for the normal development and function of female reproductive tissues. These hormones exert their functions in the target tissues through their specific intracellular receptors, the estrogen receptors (ER), the progesterone receptors (PR) and the androgen receptor (AR), which belong to the nuclear re-

ceptor (NR) superfamily and function as transcription factors to regulate the target gene expression [5]. The abnormal expression or function of these receptors has been implicated in tumors of reproductive organs in both genders. Furthermore, the development of resistance to hormonal replacement therapy for either breast or prostate cancers is also related to aberrant expression, mutation of the genes and abnormal functioning of the respective steroid receptors.

As members of the NR superfamily, the sex steroid receptors, ER, PR and AR, share the characteristic structure with other nuclear receptors (NRs): an amino-terminal activation, AF-1 (A/B domain); the DNA-binding domain (DBD) (C); a hinge region (D domain); and a carboxy-terminal ligand-binding domain (LBD) (E), which contains a second activation function, AF-2 [5]. In the absence of hormones, NR is sequestered in a non-productive form associated with heat shock proteins and other cellular chaperones. In this state, NR is inactive and unable to influence the transcription rate of its target gene promoters [5,6]. Upon binding with the cognate hormones, the receptors undergo a series of events, including conformational changes, dissociation from heat shock protein complexes, dimerization, phosphorylation, and nuclear translocation, which enables their binding to hormone-response elements (HREs) within the regulatory regions of target genes [5,6]. The binding of hormones to the HREs causes the recruitment of coactivators and basal transcription machinery, leading to the upregulation of target gene transcription.

Coactivators are factors that can interact with NRs in a ligand-dependent manner and enhance their transcriptional activity. Corepressors are factors that interact with NRs, either in the absence of hormone or in the presence of antihormone, and repress their transcriptional activity. Both types of coregulators are required for efficient modulation of target gene transcription by steroid hormones. Therefore, changes in the expression level and pattern of steroid receptor coactivators or corepressors, or mutations of their functional domains can affect the transcriptional activity of the steroid hormones and hence cause disorders of their target tissues. This review will summarize our current understanding about the roles that the coactivators and corepressors may play in the development and progression of cancers in both male and female reproductive tissues.

The SRC family

The SRC (steroid receptor coactivator) family is composed of three distinct but structurally and functionally related members, which are named SRC-1 (NcoA-1), SRC-2 (TIF2/GRIP1/NcoA-2), and SRC-3 (p/CIP/RAC3/ACTR/AIB1/TRAM-1), respectively [5]. Sequence analysis of SRC proteins identified a basic helix-loop-helix (bHLH) domain and two Per-Arnt-Sim (PAS) domains in the amino-terminal region, a centrally located receptor-interacting domain (RID) and a C-terminal transcriptional activation domain (AD). The bHLH/PAS domain is highly conserved among the SRC members and it serves as a DNA binding and protein dimerization motif in many transcription factors. Detailed analysis revealed three conserved LXXLL motifs (NR box) in the RID, which appear to contribute to the specificity of coactivator-receptor interaction. Histone acetyltransferase (HAT) activity was identified in the C-terminal region of SRC members and there also exist activation domains that can interact with the CREB-binding protein (CBP). The members of the SRC family interact with steroid receptors, ER, PR and AR, and enhance their transcriptional activation in a ligand-dependent manner [5].

SRC-1 was the first coactivator for the steroid receptor superfamily that was cloned and characterized [7]. SRC-1 is a common transcription mediator for nuclear receptors, functioning through its HAT activity and multiple interactions with agonist-bound receptors. SRC-1 exhibits a broad range of specificity in the coactivation of the hormone-dependent transactivation of nuclear receptors, including PR, ER, GR (glucocorticoid receptor), TR (thyroid hormone receptor), and AR. Targeted deletion of SRC-1 gene in mice has indicated that SRC-1 is required for efficient steroid hormone action in vivo; for estrogen and progesterone action in the uterus and mammary gland, and for androgen action in the prostate and testis [8].

The role of SRC-1 in the development or progression of cancers is not clear. Although it is an important coactivator for ER and PR, there have been no positive results showing that the expression of SRC-1 is altered in breast cancers or ovarian cancers. However, results from different groups indicated that SRC-1 is involved in the progression of prostate cancers. Using RT-PCR (reverse transcriptase-polymerase chain reaction), Fujimoto and colleagues found that the expression levels of SRC-1 were higher in higher grade prostate cancers or cancers with a poor response to endocrine therapy [9]. At the same time, Gregory *et al* reported that SRC-1 expression was elevated, together with the expression of AR, in recurrent prostate cancers [10]. Gregory *et al* found that SRC-2 is also overexpressed in recurrent prostate cancers. Overexpression of SRC-1 and SRC-2 confers on AR an increased sensitivity to the growth-stimulating effects of low androgen concentrations. This change may contribute to prostate cancer recurrence after androgen deprivation therapy.

SRC-3 is the most distinct among the three members of SRC family; it coactivates not only the nuclear receptors but also other unrelated transcription factors such as

those in the cAMP or cytokine pathways. Compared with the widespread expression of SRC-1 and SRC-2, expression of SRC-3 is restricted to few tissues, including the uterus, the mammary gland and the testis [11]. Disruption of SRC-3 gene in mice causes severe growth and reproductive defects, such as the retardation of mammary gland development [12]. Amplification and overexpression of SRC-3 in human breast and ovarian cancers have been observed [13–17]. Bautista *et al* reported that the AIB1 (SRC-3) amplification/overexpression was correlated with ER and PR positivity [14]. However, Bouras *et al* found that SRC-3 had an inverse correlation with steroid receptors, but a positive correlation with HER-2/Neu and p53 expression [17]. Despite of the conflicting results, the overexpression of SRC-3 in breast and ovarian tumors indicates that SRC-3 is an important factor in the tumorigenesis of the mammary gland and ovary. There is no clear evidence about the possible roles of SRC-3 in prostate tumor development and progression.

SRA/SRAP

The steroid receptor RNA activator (SRA) is a unique coactivator for steroid receptors, PR, ER, GR, and AR. Differing from the other coactivators, SRA was found to function as a RNA transcript instead of as a protein [18]. Besides, SRA existed in a ribonucleoprotein complex containing SRC-1 and it mediated transactivation through the AF-1 domain located at the N-terminal region of nuclear receptors, distinguishing it from the other coactivators [18].

SRA is expressed in normal and malignant human mammary tissues [15,19]. Compared with the adjacent normal region, elevated expression of SRA was found in breast tumors [15]. Although it is currently unknown whether the expression of SRA is correlated with that of PR or ER, the increase in the SRA levels in tumor cells may contribute to the altered ER/PR action, which is known to occur during breast tumorigenesis.

Recently, Kawashima *et al* reported the cloning and characterization of a novel steroid receptor coactivator from a rat prostate library [20]. The nucleotide sequence of this coactivator has 78.2% identity to that of human SRA, however, the cDNA of this coactivator can be transcribed into a functional protein and exerts its coactivation function as a protein instead of an RNA transcript [20]. Therefore, it was designated as steroid receptor activator protein, SRAP. Kawashima *et al* demonstrated that SRAP could enhance the transactivation activity of AR and GR in a ligand-dependent manner. The mRNA of SRAP was expressed in all the rat prostate cancer cell lines examined, while that of SRA was expressed in all the human prostate cancer cell lines. The expression level of SRA is higher in androgen-independent PC-3 cells compared with that of the androgen-dependent cell lines, DU-145 and LNCaP. Taken together, these results suggested that both SRA and SRAP play an important role in NR-mediated transcription in prostate cancer.

E6-AP/RPF1

E6-associated protein, E6-AP, and RPF1, the human homolog of yeast RSP5, are E3 ubiquitin-protein ligases that target proteins for degradation by the ubiquitin pathway. They are also characterized as coactivators of steroid receptors. It has been demonstrated by transient transfection assay that RPF1 and E6AP can potentiate the ligand-dependent transcriptional activity of PR, ER, AR, GR, and other NRs [21,22]. Furthermore, they also act synergistically to enhance the transactivation of NRs [22]. Additionally, the coactivation functions of E6-AP and RPF1 are not dependent on the E3 ubiquitin-protein ligase activity.

E6-AP is expressed in many tissues including the uterus, ovary, testis, prostate and mammary gland. It is important in the development and function of these tissues, since E6-AP null mutant mice exhibited defects in reproduction in both male and female mice [23].

The first evidence of a relationship between E6-AP and cancer was obtained from the study of a spontaneous mouse mammary tumorigenesis model. In this spontaneous model, E6-AP was overexpressed in mammary tumors when compared with normal tissues [24]. Recently, we examined the expression pattern of E6-AP in biopsy samples of human breast cancers. Our results showed that E6-AP expression was decreased in tumors in comparison to the adjacent normal tissues (Gao *et al*, unpublished data). In addition, the expression of E6-AP was inversely correlated with that of ER in breast tumors, and the decreased expression of E6-AP was stage-dependent. Interestingly, the decreased expression of E6-AP was also found in human prostate cancers (Gao *et al*, unpublished data). ER plays a major role in breast cancer development, and PR is also a target of estrogen. Thus, changes in the expression level of E6-AP, a coactivator for ER and PR, might interfere with the normal functioning of ER and PR, hence participating in the formation and progression of breast tumors. In a similar way, the altered expression of E6-AP might influence the normal functioning of AR, which plays a major role in the progression of prostate cancers.

ASC-2/TRBP/AIB3

ASC-2 (the nuclear protein-activating signal cointegrator-2), also called AIB3 (the amplified in breast cancer 3) and TRBP (TR-binding protein), has recently been characterized as a NR coactivator [25]. ASC-2 interacted with NRs, such as retinoid acid receptor (RAR), TR, ER, and GR, and stimulated the ligand-dependent and AF2-dependent transactivation of the NRs either alone or in conjunction with CREB-binding protein (CBP)/p300 and SRC-1. Sub-

sequent study showed that ASC-2 also interacted with SRF (the serum response factor), AP-1 (the activating protein-1), NF-κB (the nuclear factor-κB), and potentiated transactivation by these mitogenic transcription factors [26]. This suggests that ASC-2 is a multifunctional transcription integrator molecule.

ASC-2 is likely involved in the tumorigenesis of mammary gland, because it is amplified and overexpressed in human breast cancer specimens as well as in all the human breast cancer cell lines examined. Moreover, it may also regulate cellular proliferation or tumorigenesis by the direct interaction with SRF, AP-1 and NFκB.

L7/SPA

L7/SPA, L7/switch protein for antagonists, is a 27 kDa protein containing a basic leucine zipper domain. L7/SPA is an antagonist specific transcriptional coactivator because it can only potentiate the partial agonist activity of some antagonists, including tamoxifen and RU486, but has no effect on the agonist-mediated transcription [27]. The study by Graham *et al* indicated that the relative levels of the coactivator, L7/SPA, vs. the corepressors, which suppress the partial agonist activity of tamoxifen or RU486, might determine whether the agonist or antagonist effects of these mixed antagonists predominate in a tissue or tumor [28]. This unique property of L7/SPA could partially explain the development of resistance to hormone therapy for breast cancers.

ARAs

ARAs, androgen receptor-associated proteins, is a group of factors that can bind to AR and modulate its transcriptional activity. Based on their molecular weights, these factors were named ARA70, ARA160, ARA54, ARA55, ARA267 and ARA24.

ARA70, which has a molecular weight of 70-kDa, is also named as RFG (RET fused gene) and ELE1. ARA70 was first described as an AR-specific coactivator by Chang's group in 1996 [29]. In that report, ARA70 was demonstrated as a factor, which specifically interacts with AR and enhances the transcriptional activity of AR in response to the stimulation of androgens, including testosterone and dihydrotestosterone, but not the antiandrogen, hydroflutamide (HF). Later, it was reported that ARA70 could also interact with and facilitate the agonist activity of antiandrogens, including cyproterone (CPA), HF, and bicalutamide (casodex) [30]. Recent studies by other groups showed that ARA70 was not a specific coactivator for AR; it could also interact with PR or GR [31,32]. However, studies on the expression patterns of ARA70 in different cell lines and human cancer samples showed that the expression of ARA70 was decreased in prostate cancer [31,33–35] and breast cancer, [36] while it was increased in ovarian cancers. In breast, loss of ARA70 protein expression was found in 60% of HER2 positive breast cancers, while only 33% of HER2 negative breast cancer samples lost the expression [36]. Since androgen plays an inhibitory role for breast cancer cell growth, and HER2 stimulates the growth of breast cancers, loss of the expression of AR and/or ARA70 in breast might confer a growth advantage to these cells. In prostate, ARA70 mRNA is highly expressed in the normal epithelial cells, while benign prostatic hyperplasic and cancer cell lines express either lower or no ARA70 [36]. Methylation might be responsible for the lack of expression of ARA70 in some prostate cancer cells such as DU145 [36]. The expression of ARA70 in prostate cancer cells seems to be regulated by both ER and AR, since the prostate cancer cell line, PC-3, responded to estrogen/androgen and their respective antagonists differently in the parental PC-3 cells (AR-negative) and its derived AR-positive cells.

Other members of the ARA group, such as ARA54, ARA55, ARA24, ARA160, and ARA267 were also implicated in prostate tumors [35,37–40]. The expression of these coactivators was more or less altered in human prostate cancer cell lines or biopsy samples. However, the exact roles of these factors in prostate tumorigenesis need to be determined.

The PIAS family

The PIAS (protein inhibitor of activated signal transducer and activator of transcription) family is composed of a group of proteins that share a high sequence homology [41]. The first member of this family, PIAS1, was characterized as a coactivator for AR [42]. Through its N-terminal LXXLL motifs, PIAS1 interacted with and coactivated the AR transcriptional activity in a ligand-dependent manner [42]. Besides, PIAS1 could also modulate the activities of steroid receptors such as GR, PR and ER [42,43]. PIAS1 was expressed predominantly in the testis [42]. Furthermore, overexpression of PIAS1 was found in 33% of the prostate cancer samples examined [35]. These data suggested a possible role that PIAS1 may play in normal or cancer development of the testis or prostate.

Another important member of the PIAS family is called PIASxα or ARIP3 (AR-interacting protein 3). PIASxα/ARIP3 is similar to PIAS1 in that it is also expressed predominantly in the testis, and functions as a coactivator for AR [43,44].

SNURF

The small nuclear RING finger protein, SNURF, was identified in a yeast two-hybrid screening using the DBD of AR as a bait [45]. SNURF interacted with AR, GR and PR, and enhanced their transcriptional activity in a ligand-dependent fashion. It also potentiated the basal transcrip-

tion from steroid-regulated promoters [45]. SNURF is a nuclear protein. The expression of SNURF was relatively high in the brain, but low in the testis, prostate, seminal vesicles, spleen and kidney [45]. Moreover, the nuclear localization signal (NLS) in SNURF was found to be able to facilitate the nuclear import and export of AR [46], which is important for normal functioning of AR transactivation.

BRCA1

BRCA1 is a breast cancer susceptibility gene, and its inherited mutations are correlated with an increased risk of breast and ovarian cancers [47]. The role of BRCA1 in cancer development is quite complex. On one hand, BRCA1 was shown to coactivate p53, modulate p300/CBP expression, and function as a ligand-independent corepressor for ER, PR, and AR [48–50]; on the other hand, it was shown that it could enhance the ligand-dependent AR transactivation in both breast and prostate cancer cell lines, especially in the presence of exogenous SRC family members [51]. These results are somewhat controversial regarding the influence of BRCA1 on AR activity. ER and PR play key roles in breast cancer development and progression, and AR signaling in the breast has protective effect. Thus, it is reasonable to speculate that the normal expression of BRCA1 probably protect the breast from tumorigenesis by suppressing the ER and PR signaling pathway and promoting the AR activity. Mutation of the BRCA1 gene, therefore, increases the risk of developing cancer.

In a recent study by Ko *et al.* a genomic transcript, GT198, that mapped to the human breast cancer susceptibility locus (17q12-q21), was characterized as a coactivator for nuclear receptors such as AR, ER, PR, GR, etc [52]. GT198 has a tissue-specific expression pattern; it is expressed highly in testis, moderately in thymus, spleen, and pituitary, and hardly detected in other tissues. The role of this novel coactivator in cancers of testis or breast needs to be explored.

CBP/p300

CREB-binding protein (CBP) was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein. p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties. Both of them function as coactivators for multiple NRs as well as p53 and NF-kB; both possess intrinsic HAT activity and both can recruit HAT and p/CAF (CBP/p300-associated factor) [5]. Besides, CBP/p300 interacts with members of SRC family and synergizes with SRC-1 in the transactivation of ER and PR [53]. Based on its wide expression and multiple functions, it is speculated that CBP/p300 might participate in the process of tumor initiation and progression.

N-CoR/SMRT

N-CoR and SMRT are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with the nuclear receptors through the RIDs located in the C-terminal portion of the proteins, while their transcriptional repression domains were mapped to the N-termini [54]. N-CoR/SMRT also associates with HDAC3 (histone deacetylase 3) in large protein complexes, which is an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the NRs either in the absence of agonists (in the case of TR and RAR), or in the presence of antagonists (in the case of steroid receptors) [54]. As mentioned above, corepressors, N-CoR and SMRT, can suppress the partial agonist activity of antagonists, counteracting the effects of L7/SPA. The alteration of the expression of these corepressors changes the balance of corepressors to coactivators that are bound to the transcription complex via the antagonist-occupied steroid receptors. This might determine whether the outcome is inhibitory or stimulatory, and therefore determine whether tamoxifen-resistance will occur or not.

Other coregulators

In addition to the above-mentioned coactivators and corepressors, there are many other factors that have been characterized as sex steroid receptor coregulators. These include HMG-1/2 (the chromatin high-mobility group protein-1, 2), TIP60 (Tat-interacting protein), PNRC1/2 (proline-rich nuclear receptor coregulatory protein-1, 2), Cdc25B, Uba3 (ubiquitin-activating enzyme 3), and RTA (repressor of tamoxifen transcriptional activity) [55–60]. At present, it is not clear whether these coregulators are involved in the development of cancers.

Conclusion

Steroid receptors activate their target gene transcription in response to the hormonal stimulus. Their transactivation activities are modulated by coregulators (coactivators and corepressors). Different coregulators exert their actions through different mechanisms. Involvement of coregulators in the development and progression of cancers is complex. Most of the steroid receptor coactivators and corepressors identified so far are widely expressed. They usually can modulate the transactivation of multiple receptors. On the other hand, the transactivation function of a single nuclear receptor in certain tissues is usually regulated by multiple coregulators. Much evidence supports the importance of coregulators in tumorigensis and the development of hormone-resistance in breast or prostate cancers. The understanding of the mechanisms of the actions of these coregulators will be helpful for the development of new cancer therapies.

Authors' contributions

XG and BWL drafted the manuscript and ZN supervised and performed the final editing. All authors read and approved the final manuscript.

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